BEHAVIOR OF ENZYMES IN REVERSE MICELLES IN NON - AQUEOUS SOLVENTS

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By

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to the

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DEDICATED TO

MY

FAMILY

STATEMENT

I hereby declare that the matter embodied in this thesis entitled "**BEHAVIOR OF ENZYMES IN REVERSE MICELLES IN NON-AQUEOUS SOLVENTS**" is the result of investigations carried out by me in the Department of Chemistry, Indian Institute of Technology, Kanpur, India, under the supervision of Professor Sarvagya S. Katiyar.

In keeping with scientific tradition, due acknowledgement has been made wherever the work described is based on the finding of other investigators.

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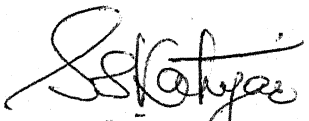

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ANIL KUMAR

SYNOPSIS

Enzymes are functional units of cell metabolism. They are highly specific and extremely efficient catalysts. In vitro studies of enzymes are usually carried out in water (in buffered medium). However, in the living cell, enzymes either act on the surface of biological membranes or inside them. In principle, properties of water near a water/membrane interface are significantly different from those of bulk water. To simulate in vitro condition of enzyme action in vivo, enzyme reactions are often performed in mixtures of aqueous and non-aqueous (apolar) solvents. But apolar environments are known to inactivate and denature the enzyme rapidly. In order to search an alternative medium for in vitro study of enzyme action in cellular reaction and to extend the utility of enzymes in technological processes, specially in organic synthesis,which are thermodynamically favoured in apolar solvent, it will be necessary to use them in apolar medium. To attain this goal, enzymes must be protected from the harmful effects of apolar environments by some means. A reasonable way out of this situation is to entrap the enzymes in aqueous inner core viz. waterpool, of the reverse micelles formed by the surfactant aggregation in non-aqueous solvents. Waterpool of the reverse micelle behaves as microreactor where guest enzyme molecules are protected from the hostile action of organic solvents. In recent years reverse micelles have been shown to

provide unique environment for enzymes, nucleic acids and other biomolecules.

First chapter of the thesis presents a brief and critical review of the relevant literature in the area of normal micelles and reverse micelles. Special emphasis has been given to the study of enzymes and their biotechnological applications in reverse micellar medium (microheterogeneous medium) because of similarity to the system chosen by us.

In the second chapter, the solubilization and activity of the enzymes in reverse micellar medium have been described. Since very few enzymes which were very simple and having low molecular weight and single subunit, were studied in reverse micelles, therefore for the study of the behavior of enzymes in reverse micelles a class of oxidoreductase enzymes has been chosen. A majority of oxidoreductase enzymes are also called as dehydrogenases or reductases. The idea to select the enzymes of this class was based on their diverse characteristics such as having more than one subunit, large and complex enzymes, involved in coupled reactions and enzymes having very specific and important use in many biological and technological processes. Dehydrogenases are involved in various metabolic mechanisms in the living cells. Moreover dehydrogenases are the key to many biological processes. Therefore, investigations on dehydrogenases in such kind of microheterogeneous medium are likely to provide a realistic picture of enzyme behavior as the hydrophobic and polar environment provided by reverse micelles is

somewhat similar to the cellular environment. In this context following three enzymes have been studied extensively.

(i) Dihydrofolate reductase (DHFR) or Tetrahydrofolate dehydrogenase

(ii) Malate dehydrogenase (MDH)

(iii) Lactate dehydrogenase (LDH)

These enzymes have been selected keeping in mind their important biological and technological characteristics. They are in increasing M.W. and complexity from DHFR-MDH-LDH direction. Dihydrofolate reductase is a key enzyme in folate metabolism and the primary target for antifolate drugs. The enzyme catalyses the reduction of 7,8-dihydrofolate to its active form tetrahydrofolate.

$$\text{Dihydrofolate} + \text{NADPH} + H^+ \xrightarrow{\text{DHFR}} \text{Tetrahydrofolate} + \text{NADP}^+$$

This enzyme is of low molecular weight (M.W.$\approx$ 20000 dalton) but it is very important in terms of medical point of view. The enzyme is strongly inhibited by certain drugs such as methotrexate (an analog of dihydrofolate) clinically useful in the treatment of some forms of cancers.

Malate dehydrogenase is relatively bigger and complex enzyme (M.W.$\approx$ 70000 dalton, 2 subunits) than DHFR. MDH is an enzyme of citric acid cycle and it catalyses the last reaction of this cycle.

$$\text{Malate} + \text{NAD}^+ \overset{\text{MDH}}{\rightleftharpoons} \text{Oxaloacetate} + \text{NADH} + H^+$$

This enzyme is used in coupled assay for the determination of activity of other enzymes such as ATP-citrate lyase, citrate synthase etc. It is of clinical interest as its activity in serum has been shown to be of diagnostic significance.

The next enzyme of increasing M.W. and complexicity is lactate dehydrogenase. This is a bigger enzyme (M.W.$\approx$140,000 dalton and 4 polypeptide chains) than MDH and much bigger than DHFR.LDH plays an important role in several metabolic pathways. It forms the centre of a balanced equilibrium between catabolism and anabolism of carbohydrates. In anaerobic glycolysis, LDH is the terminative enzyme in the sequence of reactions that promote the breakdown of glucose to lactate therefore it is essential for the production of ATP, an efficient energy carrying system in cells. LDH catalyses the following particular reaction

$$\text{Pyruvate} + \text{NADH} + H^+ \overset{\text{LDH}}{\rightleftharpoons} \text{Lactate} + NAD^+$$

The function of LDH is to regulate pyruvate lactate equilibrium in the living cells. In vitro, LDH is used in the coupled assay for the determination of activity of other enzymes such as pyruvate kinase, myokinase etc. It is also used for the determination of lactate concentration in blood or serum.

For the study of these enzymes in reverse micelles, the system cationic surfactant cetyltrimethylammonium bromide (cetrimide, CTAB) in isooctane-chloroform (1:1, v/v) non-aqueous medium has been used. With few exceptions, anionic surfactants were found to denature dehydrogenases. These enzymes, under

specific conditions have been found to be active in the CTAB reverse micellar system. Spectroscopic perturbation of the guest enzymes is smaller in CTAB than in anionic surfactant sodium bis(2-ethylhexyl) sulfosuccinate abbreviated as aerosol OT or AOT. The choice of this surfactant system is also based on its special characteristics to provide a bigger water pool size for big molecules of enzymes. The size of the water pool can be varied by either changing water content or surfactant concentration. The molar ratio of water to surfactant concentration is called degree of hydration i.e. w_o (w_o = $[H_2O]/[CTAB]$). The w_o is an important parameter which determines most of the structural and physical properties of the reverse micelles.

Solubilization of these enzymes in reverse micellar system is governed by some parameter like w_o, CTAB concentration, type and concentration of buffer, concentration of aqueous stock solution of enzymes, temperature etc. Solubilization of enzymes and substrates in this microheterogeneous system was accomplished by the injection method. 2-10 μl of the concentrated aqueous stock solutions of the enzymes or substrates were injected with the help of microsyringe in the reverse micellar solution and the resultant mixture was vigorously shaken. The reverse micellar solutions were found to be homogeneous and optically transparent. One important characteristics of the reverse micelles is having a communication channel that permits rapid exchange of material from one micellar aggregate to another aggregate. The solubilized enzymes reside in the centre of water pool,

surrounded by shell of water molecules which protect the enzymes from the surfactant wall and from the bulk organic solvents.

The activity of enzymes was measured by the spectrophotometric method. The initial velocity of enzyme reaction was measured by observing the decrease in absorbance with time at the absorption maxima (340 nm) of NADH/NADPH and the specific enzyme activity was calculated. The enzyme activity was found to be dependent on various parameters like w_o, pH_{stock} (pH of the stock buffer solution injected into the reverse micellar solution) and surfactant concentration etc. These parameters highly regulate the enzyme activity in reverse micellar system. The maximum enzyme activity was observed at the optimum conditions (w_o, pH and CTAB concentration) and these conditions were different for different enzymes. Striking feature of the enzyme activity in this reverse micellar system, is the appearance of super activity in dihydrofolate reductase at w_o 13.33, pH 7.0 and CTAB concentration 75 mM. Super activity means higher enzyme activity of DHFR in micellar solution than that obtained in aqueous medium at its optimum condition. Lactate dehydrogenase was found to be fully active in this microheterogeneous system. At w_o 30.55, pH 7.0 and 100 mM CTAB concentration, activity of LDH was 100% of its value in aqueous buffer. Malate dehydrogenase was able to retain almost full activity compared to the activity found in aqueous medium. At w_o 25.55, pH 10.3, CTAB concentration 100 mM, the activity of MDH was 80% as compared to the aqueous medium. For these enzymes the activity profiles appear to be complex and are highly dependent

on these parameters. These data on the study of enzymes (DHFR, LDH and MDH) show that these dehydrogenases are able to retain their catalytic activity while solubilized in reverse micelles and thus maintain their conformational integrity and subunit-subunit interaction inside the micellar core. Since reverse micelles have some features similar to those of biomembranes, therefore display of superactivity by DHFR shows that enzymes *in vivo* may posses higher activity than actually found by *in vitro* studies in aqueous solution.

Third chapter of the thesis deals with the investigation carried out for establishing product formation in enzymic reaction catalysed by these dehydrogenases. This chapter also presents investigation on storage and operational stability of these dehydrogenases in this microheterogeneous system of CTAB in isooctane/chloroform/water. The identical nature of the enzyme reaction in water (buffer medium) and micellar solution has been established with the help of spectral data. For this the absorption spectra of the aqueous and reverse micellar solution, before and after the completion of enzyme reaction, were recorded. These spectra recorded at different condition of w_o, pH and CTAB concentration, established the formation of same product in both the media.

Time dependent stability of enzymes in an environment is of great interest because it can improve the storage condition of enzymes. Time dependent stability of these enzymes in this system were carried out by incubating the enzyme with or without

coenzyme or substrate at 30°C. Aliquots were taken at different time intervals and the residual activity was checked. The data show that these enzymes (DHFR, LDH and MDH) when incubated with NADH or NADPH show better stability than incubated with substrates or enzyme alone. Time stability was dependent on w_o and other parameters. DHFR shows better stability at low value of w_o (w_o = 7.22) as w_o increases enzymes showed more inactivation. For DHFR the conditions for better stability and super activity were different which probably indicates that enzyme has different conformation for stability and super activity. Both MDH and LDH show better stability in conditions where they show their maximum enzyme activity, this probably indicates that they have same conformation for stability and activity.

In the last chapter kinetic parameters and kinetic characteristics for these enzyme (DHFR, LDH and MDH) in the microheterogeneous medium have been described. Effect of substrate concentration in this medium showed that these enzymes obey Michaelis-Menten kinetics up to specific concentration range of either substrate or coenzyme. At high substrates concentrations, the enzymes exhibit substrate inhibition. Similar to the aqueous medium these enzyme in reverse micelles follow initial velocity patterns (Lineweaver-Burk plots). Michaelis constant (K_m) and other kinetic and binding parameters at different values of w_o were calculated from the primary and secondary Lineweaver-Burk plots. K_m in micellar medium can be expressed in two way (i) (K_m) overall or $(K_m)_{ov}$ (ii) (K_m)

water pool or $(K_m)_{wp}$. If concentration of substrates is considered in overall volume of micellar solution then it gives the value of $(K_m)_{ov}$ and if concentration of substrates is considered to be restricted in waterpool then it gives the value of $(K_m)_{wp}$. From secondary plot, obtained from primary lineweaver-burk plots, $(K_m)_{ov}$ has been calculated. $(K_m)_{wp}$ can then be calculated from following equation.

$$(K_m)_{ov} = (K_m)_{wp} \cdot F_w$$

where F_w is water volume fraction.

For these dehydrogenases $(K_m)_{ov}$ for both substrate or coenzyme is in good agreement with $(K_m)_{aqueous}$ (i.e. $K_m^{micelle} \approx K_m^{aqueous}$) while $(K_m)_{wp}$ is an order of a magnitude higher than $(K_m)_{aqueous}$. Since reverse micellar solution is a homogeneous solution therefore $(K_m)_{ov}$ seems to be valid K_m in micellar solution. As K_m is a good measure of dissociation constant of the enzyme-substrate (E-S) complex therefore K_m in micellar solution indicates that the stability of the E-S complex in reverse micellar media remains almost unaltered.

The results of maximum enzyme activity (full or even higher enzyme activity than those in aqueous medium), stability and kinetic characteristics of these enzymes in reverse micellar medium have been discussed in terms of special microenvironment generated by the formation of right type of surfactant aggregates wherein the enzymes retain their most active conformations.

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CHAPTER-I

GENERAL INTRODUCTION

Enzymes are of supreme importance in biological systems. Life depends on a complex network of chemical reactions brought about by specific enzymes. The striking characteristics of all enzymes are their catalytic power and specificity. They accelerate reactions by factors of at least a million. They have highly distinctive and biologically crucial properties. Enzymes do not alter reaction equilibria. Rather they serve as catalysts by reducing the activation energy of chemical reactions. The living cell is a very highly organised system; containing many complex structures like mitochondria, lysosomes, peroxisomes, ribosomes, chloroplasts, golgi apparatus, secretory granules, nucleus etc. A knowledge of various enzymes within the cell in relation to these structures is highly important for the understanding of the function of cell.[1-5]

I.1 IN VITRO STUDY OF ENZYMES

In vitro studies of enzymes are usually carried out in water

(buffer). However, in the living cell quite a number of enzymes act on the surface of biological membranes or are embedded in the inner part of the membrane. More clearly, the membranes of cell and subcellular organelles are the site of several enzymes.[6-8] It is known that properties of water in proximity to the interface differ significantly from those of bulk water.[9,10]

These reasons indicate that enzymes in vivo do not function in simple water but in an environment which is more like the water/organic medium interface. It is, therefore, considered by many researchers that traditional studies on the behavior of enzymes in aqueous solution provide an imperfect picture of biological reality.[11]

To simulate in vitro the condition of enzyme action in vivo, enzymatic reactions are often carried out in a mixture of water & organic solvent having a higher concentration of nonaqueous components. However such homogeneous media are also far from ideal. A reasonable way out of this situation is to search for adequate models that may simulate the structure and function of enzymes-containing fragments of the cells and of biological membranes.[12-14]

I.2 MODEL OF BIOMEMBRANES

Biological membranes are organised assemblies that consist of mainly proteins and lipids. Lipids are amphiphilic molecules and many enzymes are deeply embedded in the hydrophobic region of lipid bilayers. It has been thought that a model having known

lipid arrangement might be useful for the in vitro study of enzyme behavior. Lipid molecules can associate in various ways and may form reverse micellar structures which represent an extremely realistic model of biomembranes.[3,4,14,15]

The lipids which are called natural surfactants are quite expensive but synthetic surfactants are much more cheaper. Aggregation of synthetic surfactants also provides a very good medium for in vitro study of enzymes both from economic and scientific standpoint.[16,17] Association of these surfactants in water gives normal micelles whereas in organic solvents reverse micelles are formed Schematic representation of normal and reverse micelles is given in Fig. I.1. Both normal and reverse micelles are recognized models of biologically important structures.[18-20] A more detailed information about surfactants, normal micelles and reverse micelles will be given later.

1.3 USE OF SURFACTANTS IN THE STUDY OF ENZYMES

Studies on enzymes in aqueous surfactants solution (normal micelles) have resulted in solving many methodological problems like solubilization of water insoluble membrane proteins, solubilization of water insoluble substrates for enzymes etc.[8,21,22] Most membrane bound proteins can be extracted from their membranes by surfactants possessing lipophilic chains which bind to the protein at its hydrophobic regions. Sodium deoxycholate is widely used for this purpose. Sodium dodecyl sulfate (SDS) has been used in SDS gel electrophoresis which enables to determine the molecular mass and subunit composition

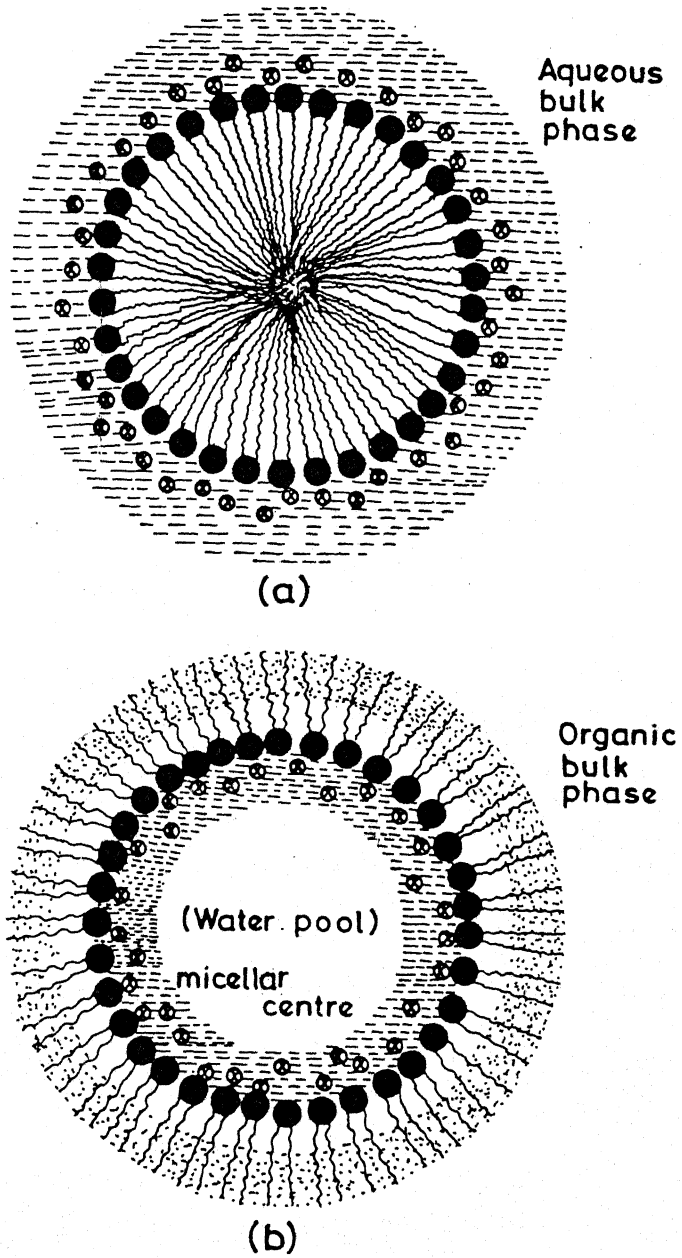


Fig.I.1. A two-dimensional schematic representation of spherical ionic (a) normal micelle (b) reverse micelle. (●) the polar head group;(⊗)the counter-ion; (≈≈≈) the hydrocarbon chain.

of proteins. Nonionic surfactants such as Triton X-100, Brij-35 are mild in their action and most proteins, whether originally membrane bound or not can maintain their activity in these surfactants. On the other hand surfactants like SDS have extremely denaturing effect on enzyme proteins. Catalytical properties of enzymes in such systems remain poorly studied. The situation can be easily explained by the fact that many enzymologists consider surfactants to be merely denaturating agents.[1,14,23]

1.4 IMPORTANCE OF THE STUDY OF ENZYMES IN REVERSE MICELLES

The tertiary system of surfactant/water/organic solvent results in the formation of reverse micelles which possess novel prospects for enzymology due to the additional component namely water-immiscible organic solvents. For a long time organic solvents have been used for the extraction of proteolipid fragment of biological membranes.[8] Proteolipid complexes in organic solvents serve as building blocks for the reconstitution of biomembranes. As for the structure of proteolipid complexes it is believed that they are similar to the reverse micelles of phospholipid with an entrapped protein.[24,25] An early study on the structure of an entrapped protein, cytochrome C, entrapped inside a phospholipiid complex in isooctane showed that the protein has same absorption spectrum and dispersion of optical rotation as it has in water or in lipid in water dispersion.[26,27] The system of phospholipids in organic solvents has been studied in detail and it was found that the phospholipids form reverse

micelles. Montal et al. established the spectral characteristics of integral protein (rhodopsin) in the phospholipid complex in hexane, are similar to those of the native state in biological membranes.[29-31] It has also been discovered that the absorption spectra of trypsin and chymotrypsin in hydrated phospholipid complex dissolved in isooctane are similar to those in water.[32]

A rapid development of enzymological research in organic solvents started in 1980's when natural surfactants (phospholipids etc) were replaced by synthetic surfactants to solubilize enzymes. Luisi in ETH Switzerland found that when α-chymotrypsin was solubilized in cyclohexane by means of cationic surfactant (N-methyltrioctyl ammonium bromide), these was almost no change in the absorption spectrum compared with water.[16] Similarly Martinek reported that when α-chymotrypsin and peroxidase were entrapped inside the reverse micelles of anionic surfactant (sodium bis(2-ethylhexyl)sulfosuccinate so called Aerosol OT or AOT) in organic solvents (Octane/Benzene), the catalytic activity was retained.[17] Now few groups are engaged in micellar enzymology in organic solvents.[14,20,31,33-35] The modern stage of micellar enzymology development is characterised by the fact that colloidal solution of water in organic solvents, stabilized with amphiphilic compounds like surfactants, represents an universal microheterogeneous medium for enzyme reactions.[36]

1.5 UTILIZATION OF ENZYMES IN ORGANIC SOLVENTS

Another important reason for the study of enzymes in reverse micelles in organic solvents is based on the utilization of enzymes as biocatalysts in many technological processes. Specific prospects are the use of enzymes as catalysts in the synthesis of fine organic chemicals, for the preparation of medicinal substances and for the synthesis of important biochemical compounds.[37-39] However, such wide spread employment of enzymes in practice is hindered by the fact they retain their unique catalytic properties usually only in aqueous solution under mild conditions. On the other hand it is known that many chemical reactions are driven by thermodynamic factors towards the required product only in specific organic solvents. This is some times due to the specific solvation effect or to the solubility of individual components of the reaction or in other cases to the fact that together with required product water is formed and consequently in aqueous solution the equilibrium is strongly shifted towards the original substances. Unfortunately change over from water as the reaction medium to an organic solvent is invariably accompained either by complete denaturation of the enzyme or in a sharp decrease of its catalytic activity and the disappearance of its substrate specificity.[13,40,41] One of the possible way out of this situation is the creation of a model catalytic system, survivable in organic solvents. In particular the system of reverse micelles in organic solvents can be used as such a model system. Enzymes can be solubilized in the polar core of the reverse micelles. The enzyme thus

solubilized retains practically completely its catalytic activity and substrate specificity. Apparently the enzymes being enclosed in the reverse micelles are protected against denaturation (unfolding of their structures) due to the fact that interface between the protein globule (or its surface layer of water) and the organic solvents phase is stabilized by the surfactant molecules. The enzyme can directly and without adverse contact with the organic solvent, exist in a unique microreactor.[17,41-43]

Because of these fundamental and applied reasons, detailed studies on "Reverse Micellar Enzymology" became a need of the present time. To carry out studies in this system understanding of different components involved such as surfactant, micelles, reverse micelles, enzymes and their applied aspects, is necessary. A brief discussion of these is presented here.

I.6 SURFACTANTS: FORMATION OF MICELLES AND REVERSE MICELLES

Surfactants commonly called detergents or surface active agents are amphiphilic substances possessing nonpolar regions (hydrocarbon chains) as well as polar region (head groups) which may or may not have charges. When the head group is positively charged, one refers to the surfactant as cationic whereas anionic surfactants have negatively charged head groups and nonionic surfactants have polar but neutral head groups. The nonpolar moiety can be of different length, contain unsaturated bond(s), and/or consist of two or more chains. Functional groups can also be incorporated into surfactants. Besides the polar heads and

hydrocarbon chains, ionic surfactants have counter ions which have opposite charge to the polar head group. Long chain alkyl ammonium halides and alkyl pyridinum halides are the most frequently used cationic surfactants. An example of this is hexadecyltrimethylammonium bromide (CTAB) which is generally adopted as a cationic surfactant in most of the studies. Metal salts of alkyl sulfates are commonly used anionic surfactants. Sodium dodecyl sulfate (SDS), most widely used anionic surfactant, is an example of this class of surfactants. Most nonionic surfactants are polyoxyethylene derivatives of compounds such as alkyl phenol and alcohols, fatty acid esters etc.[18,20,44]

As a result of association these surfactant molecules form a structurally rich variety of organised assemblies. If the association is in aqueous medium, it is referred to as micelle or normal micelle whereas reverse micelle is formed by the association of surfactants in nonaqueous medium.[18,20,45-48]

I.6.1 Micelles or normal micelles

Over a narrow range of concentration of these surfactants in aqueous solution, there is a sudden transition in the physical properties of the surfactants. This transition corresponds to the formation of aggregates or micelles and this concentration is used to define critical micelle concentration or CMC. A large number of method exist for the determination of CMC. CMC values depend on the several factors such as hydrophobicity of the hydrocarbon chain, on the nature of the polar head group and counter ion, on the type and concentration of added electrolytes

and on the net charge of the surfactant. CMC values are also affected by the temperature, pressure and added electrolytes.[18,20,49]

I.6.2 Micelles are dynamic species

Micelles are not static species but rather exist in a dynamic equilibrium. They rapidly break up and reform. The association of surfactant monomer is characterized by monomer $\rightleftharpoons$ n-mer association where n is the aggregation number which determines the size and geometry of the micelle. It is generally assumed that micelles at concentration close to their CMC are roughly spherical or ellipsoidal. The hydrophobic part of the aggregate forms the core of the micelle while the polar head groups are located at the micelle water interface in contact and hydrated by a number of water molecules. When the surfactant concentration markedly exceeds the CMC, the shape of the spherical or ellipsoidal micelle undergoes gradual changes. It elongates to assume cylindrical or lamellar structures. Micelles have average radii of 12-30 A^{o} and contain 20-100 monomers.[18,20,44]

I.6.3 Application of micelles

An important function of micelles is to provide an environment that differ substantially from that of the bulk water. Many applications of micelles are related to their ability to dissolve or solubilize water insoluble substances. A sudden increase in solubility occurs in the region of the CMC.

Solubilization is the basis of many industrial processes and is of fundamental importance in rationalizing micellar effects or reactivities.[18,50,51]

I.6.4 Micelles influence the Rates of Chemical Reactions

Rates of numerous organic and inorganic reactions are effected by micelles in aqueous solution. Catalysis or inhibition is the consequence of substrate solubilization in the micellar pseudo/phase. Rate effect can be attributed to electrostatic, hydrophobic, electrophilic and/or nucleophilic interaction with the resultant alteration of the free energy of activation for the overall process.[18,52] Many reactions have served well as model system in the investigations of micellar effects on reaction rates. The effect of micellar solution on various types of hydrolytic reactions has been investigated. Hydrolysis of esters, sulfates, phosphates, etc. in micellar media is characterized by the fact that micelles either retard or enhance the rate of hydrolysis which is attributed to the hydrophobic and electrostatic interactions.[18,20,53-57]

The effect of aqueous micelles has been investigated on many organic reactions. Cationic surfactants increase and anionic surfactants decrease the pseudo second-order rate constants for the Cannizaro reaction of benzaldehyde.[58] Other type of reactions studied in micellar media are; base catalysed hydrolysis of α,β-unsaturated ketones, synthesis and hydrolysis of benzylidine aniline thiol-thiamide exchange reaction, hemin equilibria, electrophilic coupling reactions, recemization of

biphenyl, decarboxylation of substituted carboxylate ions, radical and excited state reactions etc.[18,20] Transimination reactions of tetramethylazine-bis-barbiturate, barbituryl-azineindandione and azine-bis-indandione with semicarbazide have been also studied in micellar media in detail.[59,60] An important area of micellar investigation namely the reactions of triphenyl methane carbocations with nucleophiles have been studied extensively in Katiyar's group[61,65] and other workers.[66-69] Special attention of Katiyar's laboratory was focussed on the reactions of several triphenylmethane dyes namely, ethyl violet, methyl violet, malachite green, brilliant green, setoglaucin with nucleophiles such as OH^- and CN^- in the presence of cationic, anionic and nonionic micelles. An appropriate quantitative treatment for micellar effects and for the effects of counterions was developed.[70,71]

I.6.5 Kinetics of reactions in micelles

Interest in micellar chemistry was initially prompted by the proposed similarities between the structures of globular protein and spherical micelles and between micellar and enzymatic catalysis. Micelle catalyzed reactions can be treated in a manner analogous to enzyme catalyzed reactions. Binding constant (K_s) for the substrate micelle interaction can be determined from the values of the CMC and the aggregation number.[18,20] Mathematically micelle catalyzed reactions can be treated by the

following equation.

$$\frac{1}{k'_w+k_\psi} = \frac{1}{k'_w+k'_M} + \frac{1}{(k'_w-k'_M)K_s[M]} \quad .. \text{(I.1)}$$

where k'_w and k'_M are the first order rate constants in the bulk and micellar pseudophases, respectively and k_ψ , is observed first order rate constant for the reaction.

This equation is similar to the Lineweaver-Burk equation used in enzyme kinetics and describes the kinetics of micelle inhibited and catalyzed unimolecular processes.

I.6.6 Poor ability of micelles to mimic enzymes

Although micelles in water have provided useful models for utilizing binding energies for decreasing free energy of activation, the observed rate enhancement and specificities have been, in most cases, unimpressive. The flexibility and dynamic nature of the monomer micelle substrate system, water penetration and solubilizate induced structural changes, are the reason for the relatively poor ability of micelle in aqueous solution to mimic enzymes.[48]

I.7 REVERSE MICELLES

An alternative medium for approximating the effects of selective substrate partitioning and binding, mono and multifunctional catalysis and changes in the effective microenvironment of the reactants is provided by surfactant

aggregates in nonpolar solvents. Such surfactant aggregates have been termed reversed or inverted micelles. In contrast to normal micelle, in the reverse micelles the polar head group of the surfactant molecules are directed towards the interior of the aggregates and form a polar core whereas hydrophobic chains are exposed to the solvent. The most important difference between aqueous and reverse micelle is that in the aqueous micelle substrate do not penetrate inside appreciably whereas in the reverse micelles substrate(s), if polar, are localized in the hydrophilic cavity of the reverse micelles.[18,20,48]

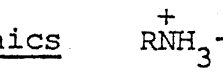
I.7.1 Surfactants used for the formation of reverse micelles

Several type of surfactants (those with large ions and small counter ions) which aggregate in apolar solvents are shown in Table - I.1 Surfactant association in apolar solvents is predominantly the consequence of dipole-dipole and ion pair interaction between the amphiphiles. This is quite different from opposing hydrophobic attraction-electrostatic repulsions responsible for micellization in water. Aggregation behavior in organic solvents is complex. It depends on the nature and concentration of the surfactant as well as on the property of the solvent. Furthermore, solubilities and aggregation of surfactants in organic solvents are dramatically affected by the presence of a deliberately added or unintentionally present third component. Experimental evidence indicates virtual impossibility of complete water removal from the surfactants. The presence of water, at least in trace amount, was, infact, suggested to be a

TABLE I.1

SURFACTANTS WHICH AGGREGATE IN NON-AQUEOUS SOLVENTS

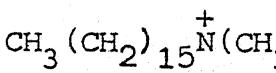
1. Cationics



$\overset{+}{R}NH_3\text{-}O_2CR'$

Alkylammonium carboxylate

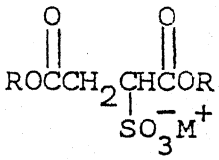
R = $C_{12}H_{25}$.R'=CH_3;DAP



$CH_3(CH_2)_{15}\overset{+}{N}(CH_3)_3Br^-$

CTAB; usually requires a cosurfactant

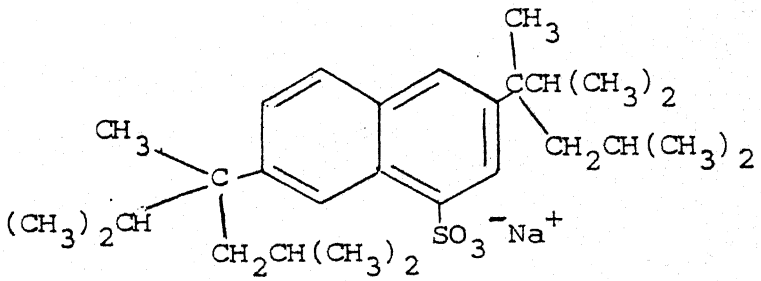
2. Anionics



sulfosuccinates

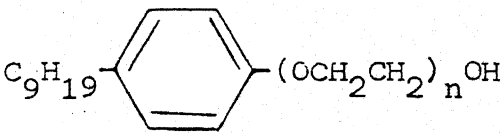
R = 2-ethylhexyl

M^+= Na^+:AOT



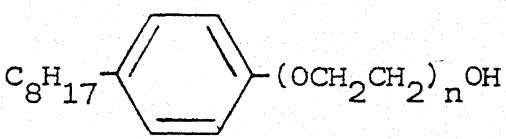
Sodium Dinonylnaphthalenesulfonates

3. Nonionics



Polyoxyethylene nonylphenols;

n = 6; Igepal CO-530

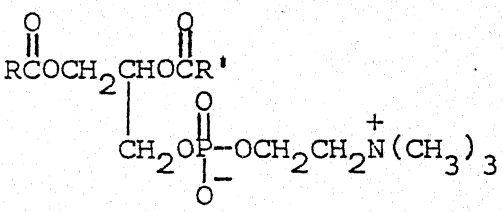


Polyoxyethylene octylphenol

n = 9.5; Triton X-100

n = 5; Triton X-45

4. Zwitterionics



Phosphatidylcholines (Lecithin)

prerequisite for surfactant aggregation in organic solvents.[18,20,47,48,72-74]

I.7.2 Association of surfactants in non-aqueous solvents

Unlike surfactant aggregation in aqueous solution, which is often characterized by well defined CMC and monomer $\rightleftharpoons$ n-mer association, surfactants in nonpolar solvents often display indefinite self association. Cationic & nonionic surfactants display stepwise aggregation behavior. (monomer $\longleftrightarrow$ dimer $\longleftrightarrow$ trimer $\longleftrightarrow$ n- mer). On the other hand anionic surfactants (Dioctylsulphosuccinate sodium salt, AOT & Dinonylnaphthalene sulfonate) and naturally occurring surfactant phosphatidylcholines (lecithin) display monomer $\longleftrightarrow$ n- mer association in nonpolar solvents.[18,20,75]

Reverse micelles, from anionic surfactants are characterized as a rule, by a narrow distribution and form which do not depend on surfactant concentration. In the case of cationic surfactants micelles are more often polydispersed and higher the surfactant concentration, higher is the portion of larger associates. The concept of CMC has been questioned for surfactants in organic solvents. It is often difficult to assign a meaningful critical micelle formation.[47] Eicke & Coworkers[76] have recently provided a general definition of reverse micelles. They considered the independence of aggregates above the operational CMC rather than their size as an essential requirement for aggregation in nonpolar solvents. Many techniques have been utilized in the

investigation on surfactant association in organic solvents. Solubilization,[77] vapour pressure depression,[76,78] U.V. absorbance,[18] dye absorbance,[79] ^1H nmr[80] light scattering,[81] surface tension methods[82] are commonly used for the determination of aggregation behavior in organic solvents. Positronium annihilation techniques have been extensively used for this purpose.[83] Shapes and sizes of surfactant aggregates in apolar solvents depend strongly on the type and concentration of surfactant and on the nature of the counterion and solvent. The linear dimension of reverse micelles are usually less than few nanometers. Aggregation number in the reverse micellar type association is very large which increases with increasing water concentration. Most important is the fact that reverse micelle exhibit relatively ordered structure characterized by a definite radius and molecular weight.[18,20]

I.7.3 Dynamic character of reverse micelles

Reversed micellar solutions are homogeneous and optically transparent. One important characteristics of reverse micelles is having a communication channel that permits rapid exchange of materials amongst several micelles. Surfactants in reverse micelles have high mobility. The Interface of the reverse micelles is well defined and nonpermeable to the organic micelles in organic solvents react equilibrium, due to their dynamic character.[33]

I.7.4 Behavior of water inside the waterpool of the reverse micelles

Due to their polar core, reverse micelles are able to solubilize large amounts of water and other polar substrates. This solubilized water is usually referred to as a waterpool. The solubilization of water produces hydrated reversed micelles significantly different (both in shape & dimension) from those formed with less amount of water. In the reverse micelle molar ratio of water to surfactant is an important parameter. This ratio is expressed as w_o (w_o = $[H_2O]/[surfactant]$) which determines most of the structural and physical properties of reverse micelles.[20,84,85] Micellar size increases as w_o increases. Indeed, for some surfactants, one may say that waterpool formation serves as a nucleating site for proper micelle formation. The properties of solubilized water depend upon w_o. Particularly at low w_o values, where the micelles are smaller and a relatively high amount of water is bound to the polar walls, water in reverse micelles behaves anomalously i.e. its physical properties are somewhat different from those of bulk water.[86] The water in the waterpool is relatively free only above a certain critical concentration. With increasing water content, the physical properties of the waterpool asymptotically approach those of pure water. However, even at relatively large w_o values, small differences may remain. With regard to the nature of water in the reverse micelles attempts are being made to improve its characterization. NMR and IR data have been obtained by a number of research groups for water in reverse

micelles. These data show that compared to normal bulk water, the bound water is motionally restricted which may have a depressed freezing point. The aqueous cavities of the hydrated reversed micelles are inhomogeneous in their physicochemical properties and are characterized by different local polarities, viscosities etc. at different points. The existence of inhomogeneity can be explained by the presence of layers of water of hydrogen bound to different extent.[33,75]

I.7.5 The phenomenon of pH in the waterpool of reverse micelles

The determination of pH of the water pool (pH_{wp}) of reverse micelles is difficult both from the conceptual and the experimental point of view. The difficulty stems from the fact that one can not reliably use a glass electrode in an organic solution containing as little as 1% water. Even if this could be done, no reliable value could be obtained, because the water of the water pool, particularly at low w_o values, is a novel solvent with unknown properties and for which no pH calibration is available. Problems are probably less severe at high water contents. In general, it appears that the pH of the reverse micelles cannot be determined with certainty. In order to estimate the acid-base properties of the micromedium in the inner cavities of the inverted micelles, the method based on comparing the pK_a of different indicators dissolved in pure water and solubilised by the inverted micelles is most widely employed. A new approach to the determination of the pH in the water pool of the reverse micelles is based on the ^{31}P NMR data. It is assumed

that the pK of the phosphate ion solubilized in AOT/isooctane reverse micelles does not change. Since ^{31}P NMR chemical shifts are sensitive to pH, the water core pH is thus determined for various AOT reverse micellar solutions by locating the observed chemical shifts on a calibration plot produced by measurements of bulk water phosphate buffer solutions. By this method, pH_{wp} was found to be generally within 1/2 pH units of pH_{st} (which is the pH of the solution to be solubilized). In fact, it probably measures the apparent pH value for the centre of the water core. Both approaches, indicator method and method based on ^{31}P NMR data, suffer, however, from significant disadvantages.[87,88]

I.7.6 Reverse micelles as novel microenvironment for chemical reactions

Reverse micellar waterpools are in dynamic equilibrium which provide unique microenvironment for interaction and reactions of polar substrates. Substrate partitioning between surfactant trapped and bulk water differential interactions, and reactivities are factors responsible for rate enhancements by reversed micelles. It is seen that rates in the presence of micelles are greater than in either the bulk organic solvents or bulk water.[18,48]

The effects of reverse micelles on a wide variety of chemical reactions (Ester hydrolysis and aminolysis, metal ion-ligand exchange, proton transfer, etc.) have been investigated.

The mutarotation of 2,3,4,6 tetramethyl-α-D-glucose is not catalyzed either by aqueous micelles or by micelle-induced bifunctional catalysts. Very substantial rate enhancements are observed, however, in benzene and in cyclohexane by micellar dodecylammonium propionate (DAP), butanoate and benzoate. The rate enhancements in these reverse micellar systems are many fold than those in pure solvent. The overall implication of these results is that the tetramethyl-α-D-glucose is oriented favourably in the micellar core when hydrogen bond formation between the dodecylammonium ion and the heterocyclic oxygen atoms can facilitate ring opening.[89]

The rate constant for the decomposition of sodium-1,1-dimethoxy-2,4,6-trinitrocyclohexadienylide in benzene in the presence of dodecylammonium benzoate, containing 0.05% DMSO (v/v), is greater by factors of 62900 and 1800 than in pure benzene or in pure water[90] respectively.

Both reversed micellar catalysis and the roles of solubilized water in reverse micellar systems have been examined for aquation, electron transfer, and isomerization reactions of chromium(III) and cobalt(III) complexes in benzene. Aquation and ligand substitution reactions of tris(oxalato)chromate(III) and cobaltate(III) anions $[M(C_2O_4)_3]^{3-}$, have been discussed in terms of one-ended dissociation. The most spectacular catalysis has been observed for the aquation of the tris(oxalato)chromium(III) anion. The aquation is up to 5 million times faster in the

restricted water pool of reverse micelle than that in bulk water.[48,91]

Reverse micellar effects on hydrolyses, and on a variety of other reactions have been investigated. 2,4-Dinitrophenyl sulfate hydrolyzes in benzene in the presence of alkyl ammonium carboxylate surfactants. Solvolysis is catalyzed both by general acid and the general base components of the surfactants in addition to the micellar catalysis. The kinetic manifestation of this reaction is a linear increase in the rate constant with increasing surfactant concentrations. Hydrolysis of p-nitrophenyl acetate (neutral) and imidazole catalyzed in the presence of DAP & AOT in benzene, toluene, octane etc with solubilized water has been investigated. Rate constants for the hydrolysis in this system at constant water and surfactant concentrations are proportional to the imidazole concentration, while at constant imidazole concentration they increase linearly with increasing ratio of $[H_2O]/[surfactant]$. It is interesting, however, that the observed constants for the hydrolysis of neutral p-nitrophenyl acetate do not exceed the value obtained in pure water.[18,48] Decomposition of p-nitrophenyl acetate in DAP and other alkyl ammonium carboxylates has been studied. Rate of reactions are affected by hydrocarbon chain length of both the alkylammonium and carboxylate groups. The dependence of reactivity in aminolysis of a series of aliphatic p-nitrophenyl esters on alkyl chain length in AOT reverse micelles is the opposite of water one finds in normal micellar system. Increasing the amount of water increased the aminolysis velocity

because the effective concentration of the ester in the reverse micelles increases.[92]

There are significant rate enhancements by the nonionic surfactant Igepal CO-530 (Polyoxyethylene (6) nonylphenol) in benzene cyclohexane carbon tetrachloride etc. Rate constants for the hydration of acetaldehyde by water solubilized in carbon tetrachloride by this surfactant are markedly greater than those in water. Hemin is solubilized by this surfactant in benzene. The equilibrium constant for the interactions of cyanide ion with heme in this system is about thousand fold greater than that in neat methanol. The rate constant for the interaction of 4-nitropyridine-N-oxide with sodium methoxide in benzene in the presence of Igepal Co-530 aggregates is many fold greater than that in neat methanol and in neat benzene.[18,48] Our group has studied the reactions of triphenylmethane dyes such as methyl violet, ethyl violet, para-rosaniline, brilliant green, malachite green etc., with nucleophiles namely hydroxide ions and phenoxide ions in the system of Igepal Co-530 in cyclohexane. The results are very interesting. The rates of reactions are enhanced by many fold (upto about thousand) than those in bulk water. Rate of reaction is highly dependent on water to surfactant ratio (w_o = $[H_2O]/[$Igepal Co-530$]$) which is maximum at lowest water pool and decreases with increasing water pool. In contrast to the bulk water and normal micelles hydroxide is a poor nucleophile in reverse micelles.[93,94]

There are many other reactions which are markedly enhanced by reverse micelles. Attempts have been made to exploit the idea that micelle-reactant interactions should be more specific in reverse than in normal micelles. Investigations of the reactions in these media can provide significant information relevant to organic and inorganic reaction mechanisms, small and large scale industrial processes and enzymatic and cell membrane interactions.[20]

I.8 ENZYMES

Enzymes are proteins whose molecular weights are in tens and hundreds of thousands. Enzymes are highly specific and extremely efficient catalysts. They enhance reaction rates 10^5-10^{10} fold in relatively dilute aqueous solutions around neutral pH at ambient temperatures.[1-5,95,96] Structure of several enzymes, their active sites, and substrate interactions these in have been determined by X-ray crystallography. Active sites of many enzymes are situated near the N-terminal end of an α-helix. The dipole field, generated by the α-helix, was suggested to play an important part in binding substrates and in enhancing reactivities.[97] The microenvironment for the substrate at the active site is quite different from that in bulk water well-recognized manifestations of this unique environment are the superactivity and the substantially altered pK_a values of functional groups in proteins. Electrostatic, hydrophobic, steric, dielectric interactions and neighboring group participation contribute to altered substrate reactivities at

enzyme active sites. They require relatively rigid conformation. However, the apparent dichotomy between rigidity and structural changes is an essential feature of enzyme functions. Several attempts have been made to account for the superior catalytic powers of enzymes in terms of known physical organic -chemical principles. Quantitative discussion of enzyme catalysis requires the selection of a set of standard reaction conditions in terms of thermodynamic standard states of free energies.[20]

I.9 SOLUBILIZATION OF ENZYMES IN REVERSE MICELLES

The importance of investigating reverse micelle solubilized enzymes are increasingly recognized. The solubility of enzymes in reverse micelles is governed by the surfactant concentration, the water to surfactant ratio (w_o), the temperature, and the type and concentration of buffers and counterions present.[20,33] Homogeneous (optically transparent, i.e. non turbid) solution of proteins and enzymes in organic solvents may be obtained by one of the following procedures:

I.9.1 Injection method[17]

The injection method is the simplest, and the one which is most commonly used for the preparation of enzyme containing reverse micelles. A few microliters of the concentrated aqueous stock solution of the enzyme is injected into the surfactant solution in an organic solvent. The resultant mixture is

vigorously shaken for few seconds until the formation of an optically transparent solution.

I.9.2 Phase transfer method[16]

This method was proposed by Luisi and coworkers. According to this method, the enzyme or protein is present initially in an aqueous solution, which is covered with a layer of micellar solution. Upon gentle stirring, a part of the protein is slowly transferred from the aqueous phase into the organic phase. This method is relatively slow, but has the advantages that the final system is in thermodynamic equilibrium and the final reverse micellar solution containing the enzyme, is generally stable.

I .9.3 Solid phase extraction method[98]

The methods 1 and 2 are appropriate for water-soluble proteins of great advantage and interest is the ability of reverse micelles to solubilize water insoluble proteins. This is accomplished according to this method, where the insoluble protein, as a powder, is gently stirred with the hydrocarbon micellar solution containing already a certain amount of water. This solubilization phenomenon can perhaps be understood on the basis of the fact that water in reverse micelles may have different physical properties from those of bulk water, so that its solubilizing ability may be changed. This method has become useful for water insoluble membrane proteins namely Folch-Pi protein (lipophylin) and more interesting, one has now a means

to study the interactions with the other protein (myelin basis protein, MBP) comprising the myelin structure, under conditions which can be considered similar to those in membrane environment.[99]

I.10 MOLECULAR MECHANISM OF THE INCORPORATION OF THE ENZYMES IN REVERSE MICELLES

In organic solvents reversed micelles provide a methodologically unique possibility to dissolve proteins of various nature under such standard conditions where the dissolved protein molecules can themselves select the microenvironment corresponding to their nature. Depending on their sizes, polarities and charges several modes of protein-reverse micelle interactions have been suggested (Fig. I.2). Hydrophilic proteins and enzymes are likely to be solubilized according to water-shell model which favours the one enzyme-per-micelle proposal. According to this model, enzyme residues in the water pool, surrounded by a shell of water which protects it from the surfactant wall and from the hostile hydrocarbon solvent.[43,100] Alternatively, highly charged proteins might be brought into solution by ion-pair formation. Ion pair interactions between the enzyme and the ionized surfactant head groups help to dissolve the protein in the water pool of reverse micelle. Proteins whose surfaces are predominantly hydrophobic might be solubilized by several small micelles (concerted micelles mechanism) or partially exposed to the organic solvent.[43] A

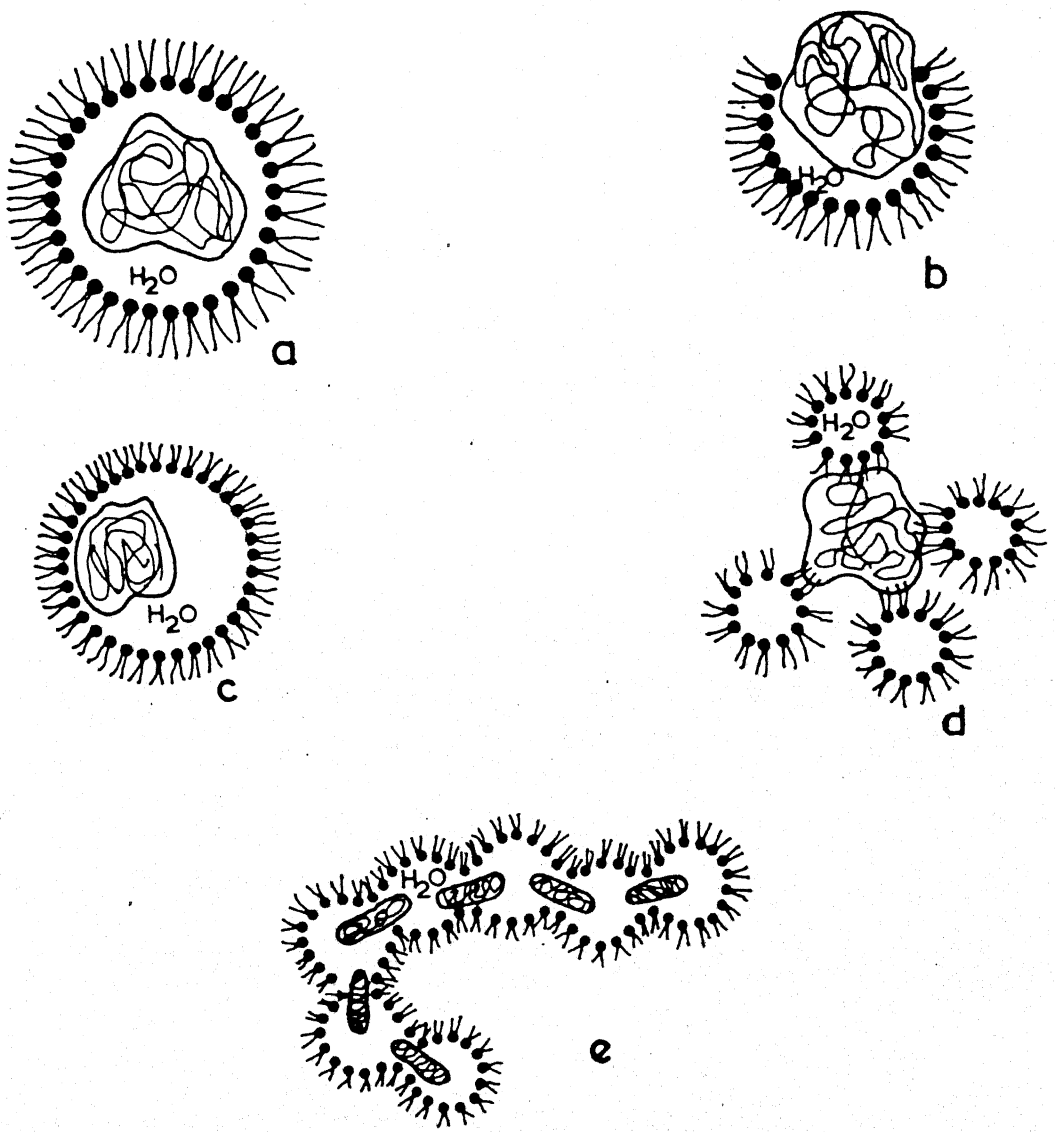


Fig.I.2. Possible models for a protein hosted in reverse micelle. (a) is the water-shell model whereby the protein is located in the water pool and is protected from the micelle wall by water layers. (b) represents the case of a protein having a very lipophilic part. (c) represents the protein adsorbed to the micelle wall. (d) is the case of a protein solubilized by the help of several small micelles (e) schematizes the formation of a network among several micelles, bridged by the protein molecules.

schematic representation of solubilization of enzyme in reverse micelles is given in Fig. I.2.

A sufficiently detailed quantitative study was made of the molecular mechanism of solubilization of water-soluble proteins. Using the water-shell model, a scheme has been developed, partly based on ultracentrifuge experiments which enables to approximate the dimensions of the protein-containing micelles and to estimate the micellar parameters characterizing such aggregates. This model was based on several assumptions, the most important being that the volume of the protein-containing micelle is the algebraic sum of the volume of the protein and the volume of water molecules originally presented in the empty micelle, and w_o does not change. The experiments gave molecular weights for the filled micelles which were, not surprisingly, higher than those of the unfilled ones. Ribonuclease, lysozyme, and liver alcohol dehydrogenase were the enzymes used in the system isooctane/AOT/water. Both sedimentation and diffusion coefficients were determined, so that molecular weights could be calculated.[100] The group of Martinek[101] has presented a model, based on ultracentrifuge studies, according to which the uptake of α-chymotrypsin does not produce any significant increase in the size of the host reverse micelles of AOT. Some other studies have also given support to structural aspects of protein containing reverse micelles using dynamic light scattering (LS) ultracentrifugation measurements and small-angle neutron scattering (SANS).

I.11 SPECTRAL CHARACTERISTICS OF ENZYMES IN REVERSE MICELLES

Protein-containing micellar solutions of water in organic solvents are optically transparent. The spectral characteristics of some enzymes and proteins, such as ribonuclease, alcohol dehydrogenase, peroxidase and cytochrome C_3, entrapped in AOT-reverse micelles in hydrocarbon solvents, are almost the same as those in aqueous solutions. The other water-soluble enzymes and proteins such as lysozyme, α-chymotrypsin, Folch-Pi-proteolipid etc. show more significant change of the spectral characteristics when they are entrapped in reverse micelles. Absorption spectroscopy, circular dichroism spectroscopy and fluorescence spectroscopy were found to be rather convenient for studying the structure of solubilized proteins and the enzyme reactions.[14,27,99,102-107]

I.12 ACTIVITY OF ENZYMES IN REVERSE MICELLAR SOLUTIONS

The fascination of reverse micellar system for the study of enzymes is that the micellar solution can be handled just like normal aqueous solution and studies on the kinetics and mechanism of enzymes are carried out easily in this homogeneous medium. In 1977 a report on the solubilization of α-chymotrypsin in cyclohexane with the help of methyltrioctyl ammonium chloride, which included the first U.V. spectrum of a hydrophilic protein in such an apolar environment, was reported. However, the enzyme was inactive.[16] A few years later, the groups of Martinek[17] and Menger[98] reported the activity of α-chymotrypsin in

hydrocarbon/AOT system,where Luisi et al. studied the activity of ribonuclease[102] micellar system. The following generalization concerning the enzyme activity in reverse micelles are possible: (1) enzymes maintain activity comparable to that found in aqueous solution, (2) there are not significant changes in the kinetic behavior, and a Michaelis-Menten behavior has been observed in reverse micellar solution, (3) the maximal activity in reverse micelles is not found at the maximal water content, but in small or medium w_o values, (4) enzymes in reverse micelles are able to accept not only water-soluble substrates, but also water-insoluble ones, (5) the stability of enzymes in reverse micelles is generally comparable to that in water, being greater at certain w_o values.[75]

I.12.1 Effect of degree of hydration on enzyme activity

One of the most striking effects observed in the study of enzymes in reverse micelle system is the dependence of the catalytic activity of the solubilized enzymes on the degree of the hydration of the reverse micelle w_o. The dependence of catalytic activity on w_o has been observed for enzymes such as α-chymotrypsin, trypsin, lysozyme, alcohol dehydrogenase, pyrophosphatase, peroxidase, alkaline phosphatase, phospholipase A_2, pancereatic lipase etc. For enzymic reactions, transferred from an aqueous solution to a system of inverted surfactant micelles, significant shifts in the pH profiles were observed.[15,33,34,36,40,41,98,103-105,108]

I.12.2 The phenomenon of super activity

Most of the enzymes solubilized in the reverse micelles either loose some fraction of their activity or retain their full activity. However, couple of enzymes such as peroxidase,[36] α-chymotrypsin[105] have been found to show a very rare phenomenon called super activity where enzyme activity becomes higher than that of the activity found in aqueous solution. The stability of some enzymes (e.g. α-chymotrypsin etc) was found to be dramatically dependent upon the degree of hydration of the surfactant. Moreover, one can choose the conditions at which enzymes retain their catalytic activity for months.[105]

I.12.3 Substrate specificity of enzymes in reverse micelles

One of the most characteristic property of enzymes is their substrate specificity. A change in the true substrate specificity has been established for some enzymes such as horse liver alcohol dehydrogenase and pancreatic lipase. Alcohol dehydrogenase from horse liver catalyses the oxidation of aliphatic alcohols to corresponding aldehydes. The optimal substrate for the aqueous solution is octanol. However, catalysis in reverse micelles shows that butanol is the best substrate in this system (AOT/octane).[108,109] The reaction of oxidation of normal aliphatic alcohols, catalyzed by ADH is given below.

$$H(CH_2)_nOH + NAD^+ \overset{ADH}{\rightleftharpoons} H(CH_2)_{n-1}CHO + NADH + H^+$$

I.13 APPLIED ASPECTS OF REVERSE MICELLES AND REVERSE MICELLAR ENZYMOLOGY

Enzymes have been widely adopted for practical use a long time ago. Nevertheless, the microhetrogeneous reaction medium based on a colloidal solution of water in organic solvents (reverse micelles), opens new prospects. Some of these are described below:

I.13.1 Organic synthesis

In fine organic synthesis, water-insoluble (or poorly soluble) compounds such as steroids, prostaglandins, alkaloids, lipids, fats etc. can be subjected to a biocatalytic action.[110] A striking example of employment of enzymes in reverse micelles for organic synthesis is the stereospecific production of steroids which has been proposed by Hilhorst and co-workers.[111] They were able to solubilize three enzymes (viz. hydrogenase, lipoamide dehydrogenase and steroid dehydrogenase) to specifically reduce a water insoluble ketosteroid to its corresponding hydroxy forms using gaseous hydrogen or the electricity as the reducing species (Fig. I.3).

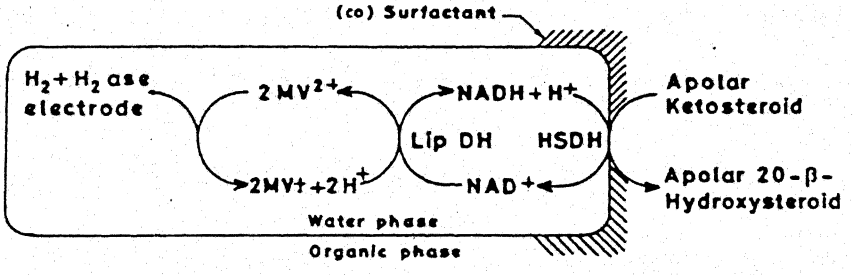


Fig.I.3. Multi-enzymic reduction of apolar steroids in a reverse micellar medium. H_2-ase, hydrogenase; lipDH, lipoamide dehydrogenase; HSDH, 20-β-hydroxysteroid dehydrogenase; MV, methyl viologen.

The enzymes are more stable in reverse micelles than in aqueous media. The product can be isolated batchwise by transferring the enzymes from the reverse micellar medium to an aqueous medium followed by precipitation of the surfactants and evaporation of organic solvent.

I.13.2 Enzymes in peptide synthesis

Enzymes have been used extensively for peptide synthesis.[112] Synthesis of water insoluble Z-Ala-Phe-Leu-NH_2 (Z is the benzyloxycarbonyl protecting group) starting with Z-Ala-Phe-OMe and Leu-NH_2 catalyzed by α-chymotrypsin take advantage of the compartmentalization of the reverse micelle (AOT/isooctane).

$$\underset{A}{\text{Z-Ala-Phe-OMe}} + \underset{B}{\text{H-Leu-NH}_2} \xrightleftharpoons{\alpha\text{-Chymotrypsin}} \underset{C}{\text{Z-Ala-Phe-Leu-NH}_2} + \text{MeOH}$$

Here B is water soluble whereas A is soluble both in water and in isooctane. The enzyme is confined in water pool. The product, C is much more soluble in isooctane than in water and after being produced in the water pool, is expelled into the external phase. Above principle can be combined with an appropriate enzyme reactor, which allows physical separation of enzyme and reagents.

Apart from AOT micelles, the reverse micelles of CTAB in chloroform-isooctane(1:1, v/v), have also been used as a medium for the synthesis of N-phenylacetyl derivatives of different peptide catalyzed by enzyme Penicillin Acylase.

$$H\text{-}Tyr\text{-}OEt + Ph\text{-}CH_2COOH \underset{Acylase}{\overset{Penicillin}{\rightleftharpoons}} Ph\text{-}CH_2\text{-}Co\text{-}Tyr\text{-}OET + H_2O$$

In addition to these reactions, there are many other reactions which are catalyzed by enzymes in reverse micelles. These reaction can not be performed (in terms of thermodynamics) in the medium other than reverse micelles.[113]

I.13.3 Solubilization of Nucleic acids, bacteria and mitochondria

Nucleic acids can be solubilized without denaturation in reverse micelles. DNA, RNA, t-RNA have been solubilized successfully in the water pool of reverse micelles. High molecular weight DNA (MW = 250,000 Dalton) contained in the reverse micelles can be visualized as suitable model for a head virus. This offers the possibility of using DNA-containing reverse micelles as models for condensed packaging of DNA *in vitro*. Studies on codon-anticodon interaction in reverse micelle are also in progress.[114]

A particularly challenging problem is the solubilization of entire cells in reverse micelles. An interesting possibility for biotechnological application is offered by the recent observation that bacterial cells can be solubilized in reverse micellar system. A reverse micellar system containing polyoxyethylene sorbitan trioleate (TWEEN 85) and water in isopropyl palmitate could solubilize whole cells of E. coli harboring, a recombinant plasmid and Acenetobacter calcoaceticus. The cells remained

viable for atleast one day and retained enzyme activity for an even longer period of time. There is a possibility of utilizing certain bacterial strains which are able to metabolize hydrocarbons and would utilize the solvent as a carbon source. Studies in this direction are in progress.[75,113,115]

The possibility of mitochondria solubilization is very important because these particles, being the chemical factories of the cells, are very interesting from the biotechnological point of view.[113]

I.13.4 The use of reverse micelles for solubilization and extraction of proteins

An example of biotechnological application of reverse micelle is separation and purification of proteins by using phase transfer method. In this method the protein is initially dissolved in aqueous solution, and a solution of surfactant in organic solvents is added as the top layer. Depending upon the salt content in the aqueous phase and other parameters, the proteins are able to leave the aqueous phase and migrate into the micellar phase. This biotechnological technique is useful in the search for rapid, inexpensive, continuous and large scale, isolation methods for enzymes. Reverse micelles can also be used for separating proteins from nucleic acids.[110,113,116]

One of the very recent finding of biotechnological aspect of reverse micellar enzymology is the purification of intracellular enzymes. In the conventional procedure after cell breakage, the

next step in the purification of intracellular enzymes is the removal of cell debris and nucleic acids by common pre-chromatography steps such as centrifugation, filtration and precipitation etc. which are rather time consuming. Use of reverse micelles replaces above pre-chromatographic procedures by two relatively simple steps. In the first step, cells (bacterial) are disintegrated by the surfactant in the reverse micellar medium and in the second step the liberated enzymes are extracted from the reverse micellar phase into an aqueous phase. A general scheme showing steps in the purification is given below. The dehydrogenases purified by this method were found to be active.[117]

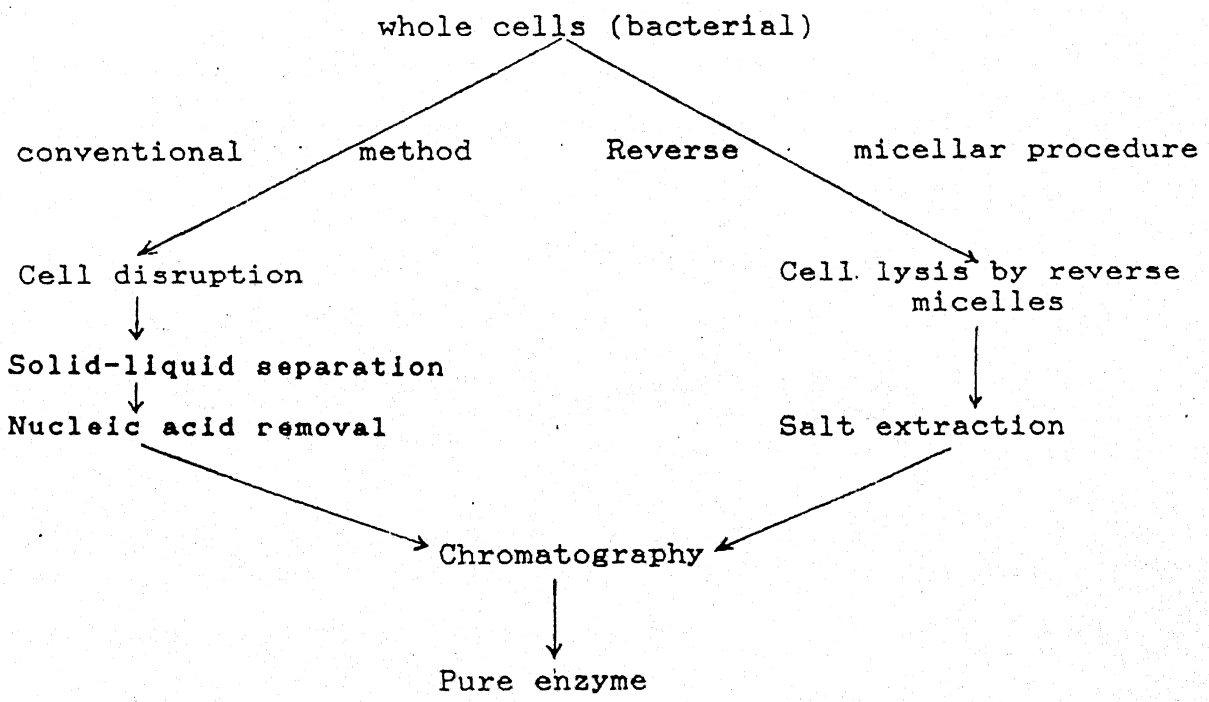


Fig. I.4

I.13.5 Cryoenzymology in Reverse Micelles

Reverse micelles provide convenient media for the investigation of enzyme catalyzed reaction at subzero temperatures surfactants stabilize the super cooled water pools surrounding the enzyme molecules against freezing due to heterogeneous nucleation. Cytochrome and trypsin were shown to retain their activities in surfactant entrapped supercooled water in organic solvents.[118,119]

I.13.6 Energy application

In order to solve the future energy problem, it will be necessary to use solar energy more extensively. The Laane et al. have demonstrated that a micellar reaction medium promoted the water biophotolysis catalyzed by hydrogenase. Reversed micelle entrapped enzyme system was shown to generate hydrogen by Ru^{2+} photosensitized electron transfer from the thiophenol donor in the organic solvent, via the methyl viologen (MeV) relay. Reverse micelles provide a microenvironment that stabilizes hydrogenase against inactivation and allows an efficient photosensitized electron and proton flow from the organic phase to hydrogenase in the aqueous phase.[42] (Fig. I.5) Shows the scheme for photosensitized production of hydrogen by hydrogenase in reverse micelles.

I.13.7 Antigen-antibody interaction in reverse micelles

The alteration in the catalytic activity of enzyme after

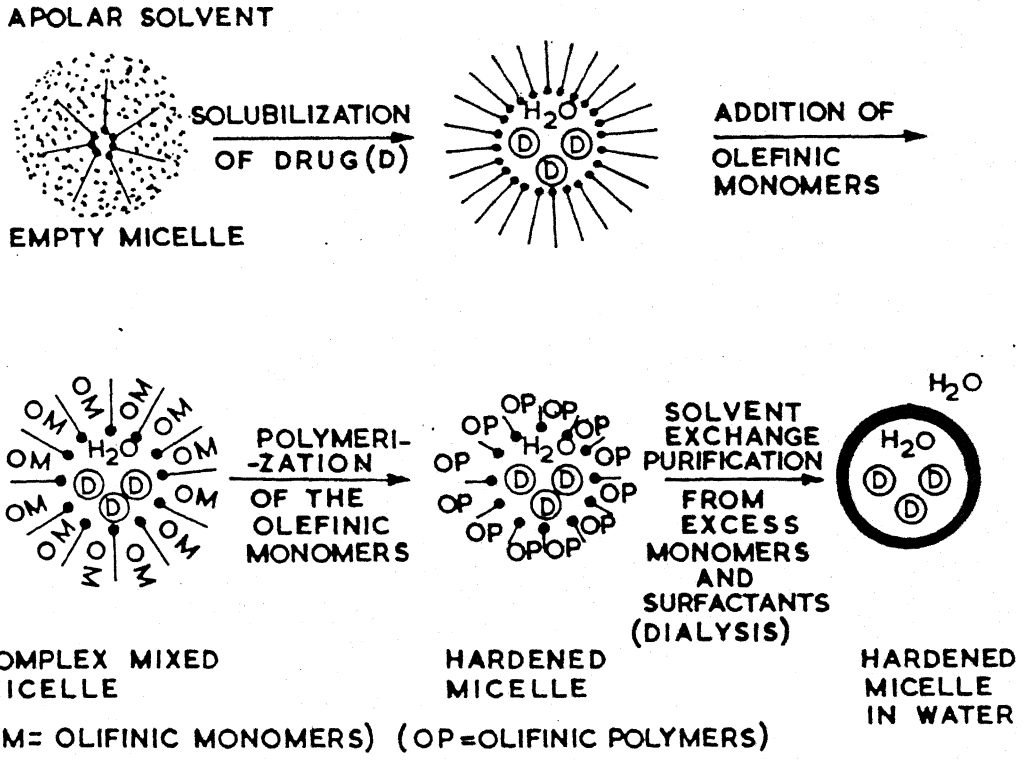
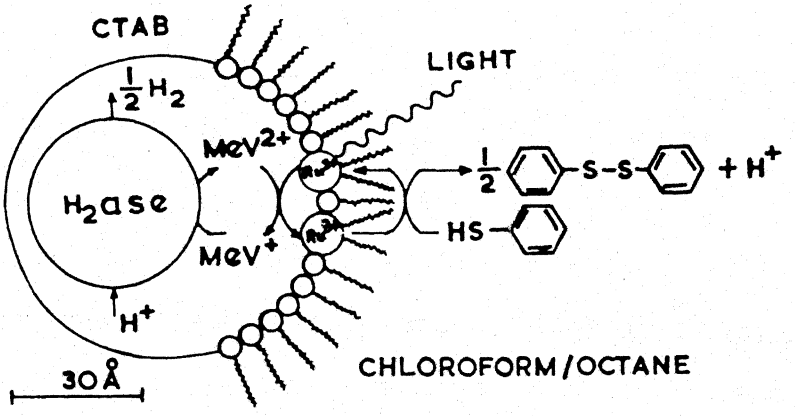


Fig.I.6. Shematic representation of the procedure for solubilization of drug into hardened reverse micelles.



(H2ase = Hydrogenase) (MeV = Methyl viologen)

Fig.I.5. Schematic representation of photosensitized production of hydrogen by hydrogenase in reverse micelles.

its interaction with antibodies against the same enzyme have been studied in reverse micelles. In the reverse micelles, the enzyme interacted with antibodies very rapidly i.e. the micelle did not hinder effective interaction between the enzyme and antibodies. The decrease in activity of the enzyme-antibody complex was dependent on w_o, pH and molarity of buffer solution as well as on the inital concentration of antibody whereas in aqueous solution it was only dependent on pH and molarity of buffer solution. Enzyme peroxidase has been studied in the reverse micellar system of AOT in heptane and in mixed micelle of AOT and Triton X-45 in heptane.[120]

I.13.8 Reverse micelles as drug delivery system

Target directed efficient drug delivery is the dream of every pharmacologist. Liposomes have been extensively used as potential drug carriers. Recently Speiser and coworkers have developed a new system of drug delivery based on reverse micelles which is known as hardened reverse micelles. The water pool of the reverse micelles hosts drug with olefinic monomers, which can readly polymerize (acrylamide) and thus form a thin resistant skin around the micelle. After the hardening has taken place, the apolar solvent is eliminated and the excess surfactant and monomer washed out. Finally hardened reverse micelles are ready to begin their function in aqueous media (Fig. I.6). Such colloidal drug carriers have many pharmaceutical uses: Immunological preparation to improve antigen-antibody response (adjuvant). This can be improved by incorporating antigen into

hardened micelles. Due to this, the protection of the antigen from degradation is high and the antibody formation is increased. These micelles can be useful in analytical measurement of pH, pO_2 and pCO_2 in body tissues; sustained and intracellular drug release to specific cells in organs etc. Reverse micelles of this type are generally referred as nanocapsules. Polymeric enzyme-containing nanocapsules also help a lot to produce new means of drug transportation in the organism.[121]

I.13.9 Gelation of reverse micelles

One of the recent finding of this field is the gelation of reverse micellar solution. The preparation of this novel family of gel is done by dissolving the gelatin in the water microphase, and following a process of warming up and cooling, the whole organic solution is transformed in a transparent gel. This gel can be practically utilized in compartmentalization of hydrophilic molecules of enzymes and/or bacteria. Enzymes can be cosolubilized with gelatin and remain active in gel form. These systems are potentially very interesting for pharmaceutical and cosmetic applications. In fact, several industries are now actively working in this area.[75,113]

I.14 OBJECTIVE OF THE PRESENT WORK

The investigations presented in this thesis were started with the aim of understanding the behavior of different class of enzymes in reverse micelles in nonaqueous solvents particularly

the investigations of dehydrogenases in reverse micellar medium. It is likely to provide a realistic picture of the enzyme behavior as the microheterogeneous environment is somewhat similar to the cellular environment.

Studies involving dehydrogenases also called reductases are of great importance because these enzymes are involved in various metabolic mechanism in the living cells such as glycolysis, citric acid cycle, vitamin and coenzyme metabolism, lipid metabolism, urea cycle etc. Moreover, these enzymes are the key to many biological processes. In our studies three enzymes namely dihydrofolate reductase (or tetrahydrofolate dehydrogenase), lactate dehydrogenase and malate dehydrogenase have been used. *In vitro* study of these dehydrogenases in reverse micellar media is of immense importance because this versatile medium is able to mimic properties of the cellular-environment *in vivo* and has a potential to extend the utility of enzymes in enzyme-mediated organic synthesis.

Till now most of the enzymes studied in reverse micelles were of low molecular weights (few thousand dalton) and single subunit. Not much attention has been paid to the enzymes having high molecular weights (hundred thousand dalton) and two or more subunits. We have solubilized such type of complex enzymes in reverse micellar water pools and investigated their characteristics.

Our studies show that these enzymes after solubilization in reverse micelles are able to retain their catalytic activity

under optimum conditions like degree of hydration (w_o), pH of the buffer solution, surfactant concentration etc. Absorption studies established the formation of same product in both aqueous and reverse micellar media. These enzymes are found to be stable in this nonpolar medium. As in aqueous medium these enzymes in reverse micelles obey Michaelis-Menten kinetics. It seems to us that reverse micellar medium provides an alternative to the aqueous medium for *in vitro* studies of these dehydrogenases which may help to extend the utility of enzymes in general in many fields of science.

REFERENCES

1. Dixon, M., Webb, E.C., Thorne, C.J.R. and Tipton, K.F. (1979) Enzymes, 3rd edn., Longman, London

2. Jencks, W.P. (1969) Catalysis in Chemistry and Enzymology, McGraw-Hill, New York.

3. Stryer, L. (1975) Biochemistry, Freeman, San Francisco.

4. Lehninger, A.L. (1984) Principles of Biochemistry, Worth, New York.

5. Fersht, A (1977) Enzyme structure and mechanism, Freeman, San Francisco.

6. Klingenberg, M. (1981) Nature (Lond) 290, 449-454.

7. Masters, C.J. (1981) CRC Crit. Rev. Biochem. 11, 105-143.

8. Maddy, A.H. (1976) Biochemical analysis of membranes, Chapman and Hall, London.

9. Cooke, R and Kuntz, I.D. (1974) Annu. Rev. Biochem. 43, 95-126.

10. Franks, F. (1975) Water - A Comprehensive Treatise, Plenum, New York.

11. Brockerhoff, H. and Jensen, R.G. (1974) Lipolytic Enzymes, Academic Press, New York.

12. Singer, S.J. (1962) Adv. Protein Chem. 17, 1.

13. Martinek, K. and Berezin,, I.V. (1977) J. Solid-phase Biochem. 2, 343.

14. Martinek, K., Levashov, A.V. Klyachko, N.L., Khmelnitski, Y.L. and Berezin, I.V. (1986) Eur. J. Biochem. 155, 453-468.

15. Misiorowski, R.L. and Wells, M.A. (1974) Biochemistry 13, 4921-4927.

16. Luisi, P.L. Henninger, F., Joppich, M., Dossena, A. and Casnati, G. (1977) Biochem. Biophys. Res. Commun. 74, 1384-1389.

17. Martinek, K., Levashov, A.V. Klyachko, N.L. and Berezin, I.V. (1978) Dolk. Akad. Nauk, SSSR (Engl. Ed.) 236, 951-953.

18. Fendler, J.H. and Fendler, E.J. (1975) Catalysis in Micellar and Macromolecular Systems, Academic Press, New York.

19. Stenius, P. (1984) in Reverse Micelles (Luisi, P.L. and Straub, B.E. eds) pp. 1-19, Plenum Press, New York.

20. Fendler, J.H. (1982) Membrane Mimetic Chemistry, Wiley, New York.

21. Tanford, C. and Reynolds, J. (1976) Biochim. Biophys. Acta 457, 133-170.

22. Helenius, A., McCaslin, D.R., Fries, E. and Tanford C. (1979) Methods Enzymol 61, 734-749.

23. Joly, M. (1965) A Physicochemical Approach to the denaturation of Proteins, Academic Press, New York.

24. Green, D.E. and Fleischer, S. (1963) Biochim. Biophys. Acta 70, 554-582.

25. Montal, M. (1974) in Perspectives in membrane biology (Estrada, O.S. and Gilter, C. eds) pp.591-622, Academic Press, New York.

26. Das, M.L. and Crane, F.L. (1964) Biochemistry 3, 696-700.

27. Ulmer, D.D. (1965) Biochemistry 4, 902-907.

28. Ohshima, A., Narita, H. and Kito, M. (1983) J. Biochem. 93, 1421-1425.

29. Ramakrishnan, V.R., Darszon, A. and Montal, M. (1983) J. Biol. Chem. 258, 4857-4860.

30. Schonfeld, M., Montal, M. and Feher, G. (1980) Biochemistry 19, 1535-1542.

31. Montal, M. (1984) in Reverse Micelles (Luisi, P.L. and Straub B.E. eds)pp. 221-229, Plenum Press, New York.

32. Austin, P., Dodd, G., Davis, M. and Leslie, R. (1974) Biochem. Soc. Trans. 2, 963-964.

33. Luisi, P.L. (1985) Angew. Chem. Int. Ed. Eng. 24, 439-450.

34. Levashov, A.V., Khmelnitski, Y.L., Klyackho, N.L. and Martinek, K. (1984) in Surfactants in Solution (Mittal, K.L. and Lindman, B. eds) vol. 2, pp. 1069-1091, Plenum Press, New York.

35. Katiyar, S.S., Awasthi, A.K. and Kumar, A. (1988) Proc. Ind. Natl. Sci. Acad. (In press).

36. Martinek, K., Levashov, A.V., Khemelnitski, Y.L., Klyachko, N.L. and Berezin, I.V. (1982) Science (Wash D.C.) 218, 889-891.

37. Whitesides, G.M. and Wong, C.H. (1985) Angew. Chem. Int. Ed. Engl. 24, 617-638.

38. Mosbach, K. (1985) in Enzymes in Organic Synthesis (Ciba Foundation Symposium III) pp. 57-70, Pitman, London.

39. Mosbach, K. (1976) Method in Enzymology, vol. 44, Academic Press, New York.

40. Levashov, A.V., Klyachko, N.L., Pantin, V.I., Khmelnitski, Y.L. and Martinek, K. (1981) Biorgan. Khim. (Eng. Ed.) 6, 485-498.

41. Martinek, K, Levashov, A.V., Klyachko, N.L., Pantin, V.I. and Berezin, I.V. (1981) Biochim. Biophys. Acta 657, 277-294.

42. Hilhorst, R., Laane, C. and Veeger, C. (1982) Proc. Natl. Acad. Sci. USA 79, 3927-3930.

43. Luisi, P.L., and Wolf, R. (1982) in Solution Behavior of Surfactants (Mittal, K.L. and Fendler, E.J. eds.) vol. 2, pp. 887-905, Plenum Press, New York.

44. Attwood, D. and Florence, A.T. (1983) Surfactant Systems, Chapman and Hall, London.

45. Mittal, K.L. (1977) Micellization, Solubilization and Microemulsions, Plenum Press, New York.

46. Mittal, K.L. (1979) Solution Chemistry of Surfactants, Plenum Press, New York.

47. Kirtes, A.S. and Gutmann, H. (1976) in Surface and Colloid Science (Matijevic, E. Ed.) vol. 8, pp. 194-295, John Wiley, New York.

48. Fendler, J.H. (1976) Acc. Chem. Res. 9, 153-161.

49. Mukerjee, P. and Mysels. K. (1971) "Critical Micelle Concentration of Aqueous Surfactant Systems", National Standards Reference Data Series, Vol. 36, National Bureau of Standards (U.S.).

50. Elworthy, P.H., Florence, A.T. and Macfarlane, C.B. (1968) Solubilization of Surface Active Agent, Chapmal and Hall, London.

51. McBain, M.E.L. and Hutchinson, E. (1955) Solubilization, Academic Press, New York.

52. Cordes, E.H. (1973) Reaction Kinetics in Micelles, Plenum Press, New York.

53. Bunton, C.A. McAneny, M. (1976) J. Org. Chem. 41, 36-39.

54. Martinek, K., Osipov, A.P., Yatsimirskii, A.K. and Berezin, I.V. (1975) Tetrahedron 31, 709-718.

55. Kunitake, T., Okahata, Y. and Sakamoto, T. (1976) J. Am. Chem. Soc. 98, 7799-7806.

56. Bunton, C.A. and McAneny, (1977) J. Org. Chem. 42, 475-482.

57. Kunitake, T. and Sakamoto, T. (1979) Bull. Chem. Soc. Jpn. 52, 2624-2629.

58. Cramer, L.R. and Berg, J.C. (1968) J. Phy. Chem. 72, 3686.

59. Reddy, I.A.K. and Katiyar, S.S. (1981) Tetrahedron 37, 655-659.

60. Reddy, I.A.K. and Katiyar, S.S. (1981) Tetrahedron 37, 585-589.

61. Patel, K.L. and Katiyar, S.S. (1978) Natl. Acad. Sci. Lett. 1, 143-145.

62. Srivastava, S.K. and Katiyar, S.S. (1982), Int. J. Chem. Kinetics 14, 1-9.

63. Malviya, S. and Katiyar, S.S. (1984), Z. Phys. Chemie Leipzig, 265, 26-34.

64. Srivastava, S.K. and Katiyar, S.S. (1980) Ber. Bunsenges. Phys. Chem. 84, 1214-1219.

65. Reddy, I.A.K. and Katiyar, S.S. (1982) in Solution Behavior of Surfactants (Mittal, K.L. and Fendler, E.J. eds.) 1017-1032, Plenum Press, New York.

66. Duynstee, E.F.J. and Grunwald, E. (1959) J. Am. Chem. Soc. 81, 4540-4542, 4542-4547.

67. Bunton, C.A. and Paik, C.H. (1976) J. Org. Chem. 41, 40-44.

68. Bunton, C.A., Carrasco, N., Huang, S.K., Paik, C.H. and Romsted, L.S. (1978), J. Am. Chem. Soc. 100, 5420-5425.

69. Funasaki, N. (1977) J. Colloid Interface Sci. 62, 336-343.

70. Malaviya, S. and Katiyar, S.S. (1981) Bull. Chem. Soc. Jpn. 54, 1852.

71. Patel, K.L. and Katiyar, S.S. (1981) Ind. J. Chem. 20A, 788-792.

72. Eicke, H.F. and Christen, H. (1978) Helv. Chim. Acta 61, 2258-2263.

73. Eicke, H.F. (1977) in Micellization, Solubilization and Microemulsions (Mittal, K.L. ed.) pp. 429-444, Plenum Press, New York.

74. Eicke, H.F. (1980) Top. Curr. Chem. 87, 85-145.

75. Luisi, P.L. and Magid, L.J. (1986) CRC Crit. Rev. Biochem. 20, 409-474.

76. Eicke, H.F. and Denss, A. (1978) J. Colloid Interface Sci. 64, 386-388.

77. Kitahara, A. (1970) in Cationic Surfactants (Jungermann, E. ed) 289, Marcel Dekker, New York.

78. Kitahara, A. (1958) Bull. Chem. Soc. Jpn. 31, 288.

79. Muto, S. and Meguro, K. (1973) Bull. Chem. Soc. Jpn. 46, 1316.

80. Fendler, E.J., Fendler, J.H., Medary, R.T. and Elseoud, O.A. (1973) J. Phy. Chem. 77, 1432-1436.

81. Kitahara, A., Kobayashi, T. and Tachibana, T. (1962) J. Phy. Chem. 66, 363-365.

82. Ray, A. (1971) Nature (London) 231, 313.

83. Jean, Y.C. and Ache, H.J. (1978) J. Am. Chem. Soc. 100, 6320-6327.

84. Eicke, H.F. and Rehak, J. (1976) Helv. Chem. Acta 59, 2383-2891.

85. Zulauf, M. and Eicke, H.F. (1979) J. Phy. Chem. 83, 480.

86. Eicke, H.F. and Kavita, P. (1984) in Reverse Micelles (Luisi, P.L. and Straub, B.E. eds.) pp. 21-35, Plenum Press, New York.

87. Elseoud, O.A. (1984) in Reverse Micelles (Luisi, P.L. and Straub, B.E. eds.) pp. 81-93, Plenum Press, New York.

88. Smith, R.E. and Luisi, P.L. (1980) Helv. Chim. Acta 63, 2302-2311.

89. Fendler, J.H., Fendler, E.J., Medary, R.T. and Woods, V.A. (1972) J. Am. Chem. Soc. 94, 7288.

90. Fendler, J.H., Fendler, E.J. and Chang, S.A. (1973) J. Am. Chem. Soc. 95, 3273.

91. O'Connor, C.J., Fendler, E.J. and Fendler, J.H. (1973) J. Am. Chem. Soc. 95, 600; (1974) J. Am. Chem. Soc. 96, 370.

92. O'Connor, C.J. and Ramage, R.E. (1980) Aust. J. Chem., 33, 757-770, 771-777, 779-784, 1301-1311.

93. Katiyar, S.S. and Awasthi, A.K. (1986)Eighth IUPAC Conference on Physical Organ. Chem. Japan. Abstract F-27-8.

94. Awasthi, A.K. and Katiyar, S.S. (1987) 3rd natl. Conference on Surfactants Emulsions and Biocolloids, India. Abstract PSS4.

95. Boyer, P.D. (1970) The enzymes, vol. 2, Academic Press, New York.

96. Kosland, D.E. Jr. (1962) J. Theo. Biol. 2, 75-86.

97. Hol, W.G.J., Van Duijnen, P.T. and Beredsen, H.J.C. (1978) Nature 273, 443-446.

98. Menger, F.M. and Yamada, K. (1979) J. Am. Chem. Soc. 101, 6731-6734.

99. Delahodde, A., Vacher, M., Nicot, C. and Waks, M. (1984) FEBS Lett. 172, 343-347.

100. Bonner, F.J., Wolf, R. and Luisi, P.L. (1980) J. Solid Phase Biochem. 5, 255.

101. Levashov, A.V., Khmelnitski, Y.L., Klyachko, N.L., Chernyak, V.Y. and Martinek, K. (1981) Ana. Biochem. 118, 42-46; (1982) J. Colloid Interface Sci. 88, 444-457.

102. Wolf, R. and Luisi, P.L. (1979) Biochem. Biophys. Res. Commun. 89, 209-217.

103. Meier, P. and Luisi, P.L. (1980) J. Solid Phase Biochem. 5, 269-282.

104. Grandi, C., Smith, R.E. and Luisi, P.L. (1981) J. Biol. Chem. 256, 837-843.

105. Barbaric and Luisi, P.L. (1981) J. Am. Chem. Soc. 103, 4239-4244.

106. Klyachko, N.L., Levashov, A.V. and Martinek, K. (1985) Molekul. Biolog. (Eng. Ed.) 18, 830-840.

107. Luisi, P.L., Bonner, F.J., Pellegrini, A., Wiget, P. and Wolf, R. (1979) Helv. Chim. Acta 62, 740, 1979-753.

108. Malakhova, E.A., Kurganov, B.I., Levashov, A.V., Berezin, I.V. and Martinek, K. (1983) Dokl. Akad. Nauk SSSR 270,474.

109. Martinek, K., Khmelnitski, Y.L., Levashov, A.V. and Berezin, I.V. (1982) Dokl. Akad. Nauk SSSR (Eng. Ed.) 263,81-84.

110. Luisi, P.L. and Laane, C. (1986) TIBTECH, 153-161, Elsevier Science, Amsterdam.

111. Hilhorst, R., Laane, C. and Veeger, C. (1983) FEBS Lett. 159, 225-228.

112. Luithi, P. and Luisi, P.L. (1984) J. Am. Chem. Soc. 106, 7285-7286.

113. Leser, M.E., Wei, G., Luithi, P., Haering, G., Hochkoeppler, A., Blochliger, E. and Luisi, P.L. (1987) J. Chim. Phys. 84, 1113-1118.

114. Luisi, P.L., Meier, P., Imre, V.E. and Pande, A., in Reverse Micelles (Luisi, P.L. and Straub;;, B.E. eds) pp.323 337, Plenum Press, New York.

115. Haering, G., Meussdoerffer, A. and Luisi, P.L. (1985) Biochem. Biophy. Res. Commun. 127, 911-915.

116. Leser, M.E., Wei, G., Luisi, P.L. and Maestro, M. (1986) Biochem. Biophys. Res. Commun. 135, 629-635.

117. Giovenco, S., Verheggen, F. and Laane, C. (1987) Enzyme Microb. Technol. 9, 470-473.

118. Douzou, P., Debey, P. and Franks, F. (1978) Biochim. Biophys. Acta 523, 1-8.

119. Douzou,, P., Keh, E. and Balny, C., Proc. Natl. Acad. Sci. U.S.A. 76, 681.

120. Eryomin, A.N., Savenkova, M.I. and Metelitsa, D.I. (1986) Biorgan. Khim., 12, 606-612.

121. Speiser, P. (1984) in Reverse Micelles (Luisi, P.L. and Straub, B.E. ed.) pp. 339-346, Plenum Press, New York.

oxidoreductases. These enzymes are involved in biological oxidation reduction reactions and therefore with respiration and fermentation processes. A majority of oxido-reductases are also known as dehydrogenases or reductases.[2]

The idea to select dehydrogenases (reductases) for the present study was based on their varied characteristics such as being made up of multi subunits, large and complex enzymes, involved in coupled reactions and having very specific involvement and use in many biological and technological processes. Dehydrogenases are not only interesting from the point of view of their specific biological function but also for their important use in preparative bio-organic chemistry. Since enzymes have been found to show different activity and specificity when entrapped in reverse micelles, the optimum conditions for maximum enzyme activity in reverse micelles are quite different for different enzymes. Therefore, investigations on dehydrogenases in reverse micellar medium are likely to provide a realistic picture of enzyme behavior as the hydrophobic and polar environment provided by reverse micelles is somewhat similar to the cellular environment. In this context following three enzymes have been studied extensively.

(i) Dihydrofolate reductase (DHFR) or Tetrahydrofolate dehydrogenase

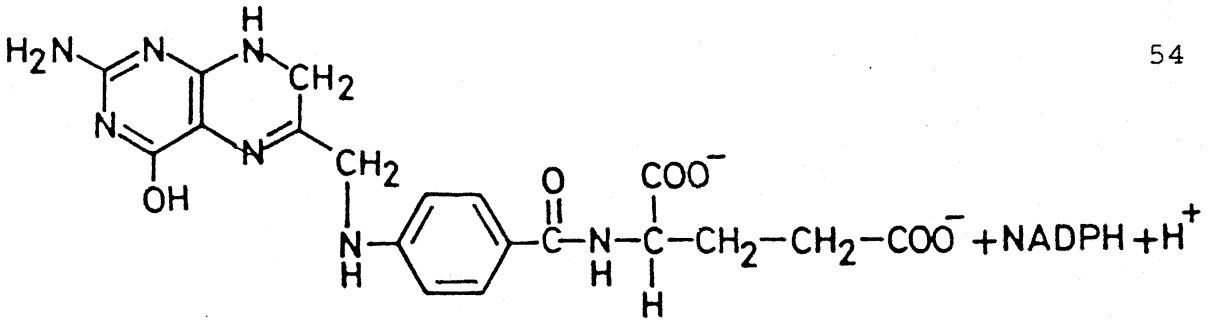
(ii) Lactate dehydrogenase (LDH)

(iii) Malate dehydrogenase (MDH)

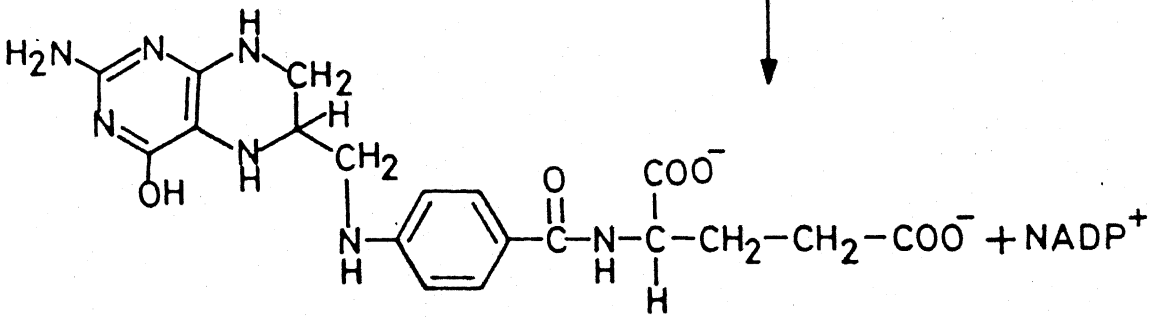
These enzymes are involved in various metabolic processes inside the cell and play very specific role in other biological processes. They have been used for diagnostic purposes in various diseases. Some inhibitors of the enzyme are used as drugs in the treatment of many diseases.[1]

Dihydrofolate reductase is a key enzyme in folate metabolism and the primary target for antifolate drugs. The enzyme catalyses the reduction of 7,8-dihydrofolate (DHF) to its active form 5,6,7,8-tetrahydrofolate (THF).[3] The overall reaction is given in Scheme II.1. It is strongly inhibited by certain drugs such as methotrexate (amethopterin, an analog of dihydrofolate) clinically useful in the treatment of some forms of cancers, acute leukemia and choriocarcinoma. The chemotherapy of this enzyme is based on its involvement in the biosynthesis of nucleotides. Since tetrahydrofolate is an essential coenzyme in the biosynthesis of thymidylic acid (a nucleotide building block of DNA) and dihydrofolate reductase represents one of the three enzymes that comprise the thymidylate synthase cycle which provide the cell a source of deoxythymidylate (dTMP) for DNA synthesis and replication, these drugs (methotrexate) inhibit the replication of DNA in susceptible cancer cells.[1,4,5]

LDH plays an important role in several metabolic pathways. It forms the centre of a balanced equilibrium between catabolism and anabolism of carbohydrates. In anaerobic glycolysis, LDH is the terminative enzyme in the sequence of reactions that promote the breakdown of glucose to lactate and therefore it is essential for the production of ATP, an efficient energy carrying system in

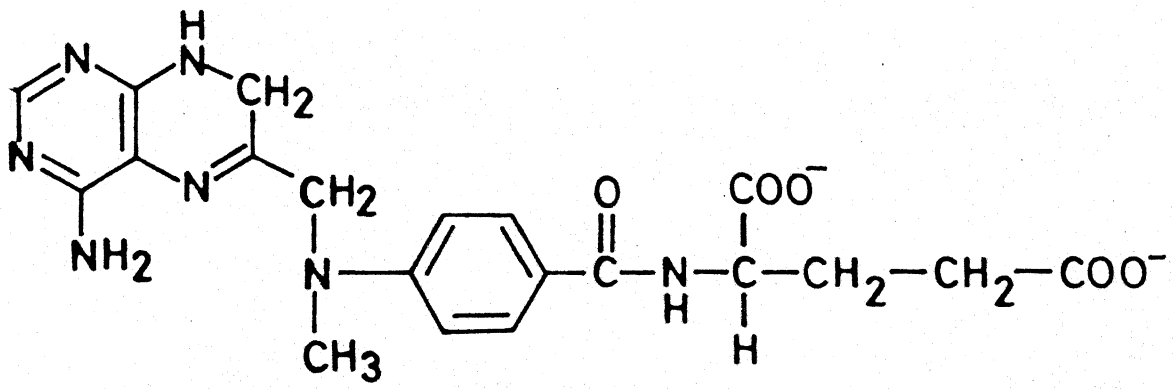


Dihydrofolate



Tetrahydrofolate

Scheme II.1. Reaction catalyses by dihydrofolate reductase (DHFR).

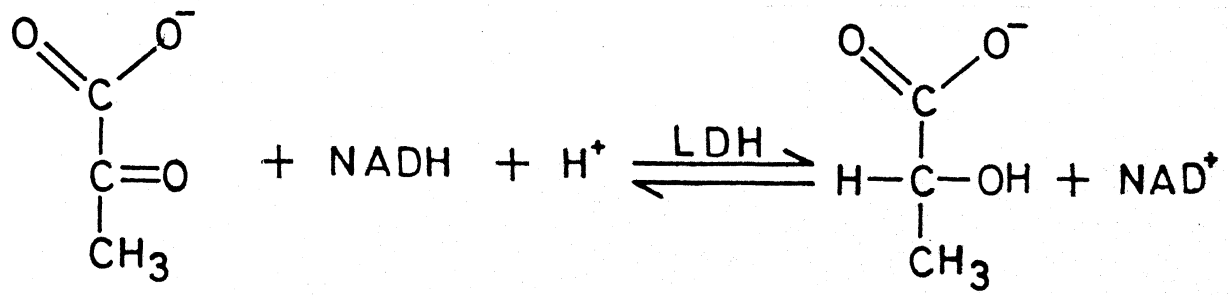


Amethopterin (methotrexate)

cells.[6] Overall reaction of anaerobic glycolysis is as follows.

$$\text{Glucose} + 2\ \text{Pi} + 2\ \text{ADP} \longrightarrow 2\ \text{Lactate} + 2\ \text{ATP}$$

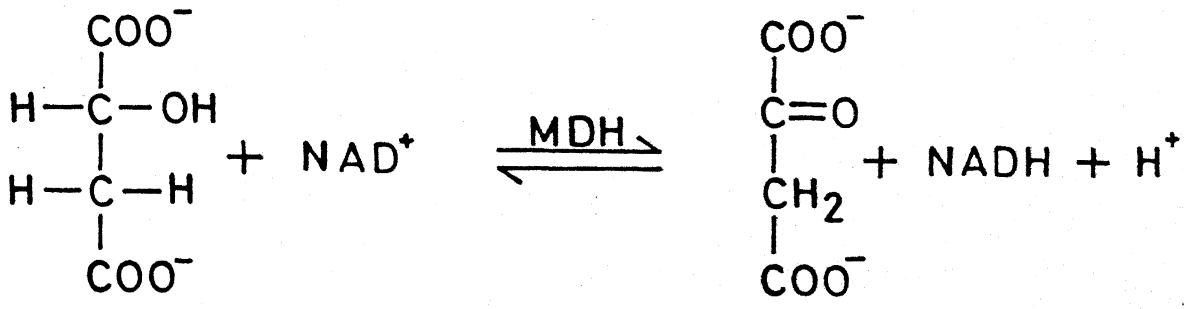
LDH catalyses the following reaction in particular.[1,4,6]



Pyruvate **Lactate**

The function of LDH is to regulate pyruvate lactate equilibrium. Mammalian LDH exists as five tetrameric isozymes which differ in catalytic, physical and immunological properties. Certain isozymes of LDH are of clinical interest as they reflect pathological conditions in particular tissues. Because of their low contaminant activities for enzymic analysis LDH from rabbit or hog muscle is preferred. In the present study LDH from rabbit muscle has been used. In vitro, LDH is used in the coupled assay for the determination of activities of other enzymes such as pyruvate kinase, myokinase, creatine kinase, enolase etc. It is also used for the determination of lactate concentration in blood or serum.[6,7]

Malate dehydrogenase is an enzyme of citric acid cycle. It catalyzes the interconversion of L-malate and oxaloacetate using nicotinamide adenine dinucleotide (NAD) as a coenzyme.[1,4,8]



Malate **Oxaloacetate**

MDH is found in all eukaryotic cells as two isozymes: mitochondrial (m-MDH) and cytoplasmic (s-MDH). Prokaryotes contain only a single form. The two enzymes are distinct, differing immunologically, structurally, catalytically and in amino acid composition. The heart enzyme is specific for L-malic acid. MDH is also used in the coupled assay for the determination of activity of other enzymes such as ATP-citrate lyase, citrate synthase etc. MDH is of clinical interest as its activity in serum has been shown to be of diagnostic significance.[9-14] In the present study mitochondrial MDH from pig heart has been used.

This chapter presents the investigations on the solubilization and activity of enzymes DHFR, LDH and MDH in the reverse micelles formed by cationic surfactant cetyltrimethylammonium bromide (Cetrimide, CTAB) in isooctane-chloroform (1:1, v/v) non-aqueous medium. With few exceptions, anionic surfactants were found to denature the dehydrogenases whereas cationic surfactants are found to provide an unique microenvironment in which the enzymes retain their maximum activity.

II.2 EXPERIMENTAL SECTION

II.2.1 Materials

Source of procurement of various chemicals and biochemicals used are described below:

II.2.1.1 Enzymes and Substrates

Bovine liver dihydrofolate reductase (EC 1.5.1.3), dihydrofolic acid, NADPH, NADH, β-mercaptoethanol and sodium pyruvate were obtained from Sigma Chemical Co. U.S.A. Rabbit muscle lactate dehydrogenase (EC 1.1.1.27), pig-heart mitochondrial malate dehydrogenase (EC 1.1.1.37) and oxaloacetic acid were purchased from Boehringer Mannheim.

II.2.1.2 Surfactant and solvents

Cetyltrimethylammonium bromide (Cetrimide, CTAB) extrapure grade reagent was obtained from SISCO Research Lab., India which was dried over P_2O_5 in an evacuated desiccator for several hours just prior to use. Isooctane puriss was purchased from Fluka and Chloroform AR from BDH.

II.2.1.3 Other chemicals

Other chemicals of analytical grade were obtained as follows: Buffer components potassium phosphates, tris, glycine, were purchased from Sigma Chemical Co. USA and potassium hydroxide, hydrochloric acid were from Merck India.

II.2.2 Preparation of enzyme and substrate reverse micellar solution

Reverse micelles containing enzymes and substrates were prepared by the injection method. According to this method, concentrated stock solutions of enzymes, substrates etc., prepared in aqueous buffer, were injected with microsyringes into the CTAB/isooctane:chloroform (1:1, v/v) solution. The desired water content was set by an additional injection of the buffer solution into the reverse micellar solutions and the resulting mixture was shaken vigorously on a vortex mixer until the formation of a homogeneous (optically transparent) solution.[15,16] The buffers used to prepare the stock solutions of enzymes, coenzymes and substrates were potassium phosphate, tris-hydrochloric acid, glycine-potassium hydroxide, tris-glycine. pH was measured on ELICO LI-120 digital pH meter. The concentrations of the enzymes, coenzymes, substrates and buffer components were adjusted according to the case of solubilization at specified water pools.

II.2.3 Enzyme activity measurement

Activity of enzymes was measured spectrophotometrically by using Gilford Response and Gilford 260 recording u.v./visible spectrophotometers. The cell compartment was kept at the $30^{o}C$ within the precision of $\pm 0.1^{o}C$ by circulating water in the thermospacer set of the spectrophotometer from an external thermostat which controlled temperature by a high precision

electronic relay. The reverse micellar solutions containing substrate, coenzyme and desired amount of buffer, were incubated for few minutes and the reaction was started by injecting (2-10 μ l) of aqueous stock solution of enzyme to the incubation mixture. The resultant micellar solution was put into the quartz cell of 1.0 cm path length and the initial velocity of enzyme reaction was measured by observing the decrease in absorbance with time at the absorption maxima (340 nm) of NADH/NADPH which gives $A_{340\ nm}$/min. Adequate control runs were carried out to ascertain the absence of artifacts like scattering and self dissociation of species in the micellar media.

II.2.4 Enzyme activity calculation

The enzyme activity is defined as μ-moles or n-moles of product formed per minute per milligram of enzyme (protein) and was calculated as follows:

By Beer - Lambert's law

$$\text{Absorbance (A)} = \text{ECl or } C = \frac{A}{EL}$$

where E = Extinction coefficient, C = concentration, l = path length in cm.

Here $E^{NADH/NADPH} = 6.2 \times 10^3$ litre/moles.cm and l = 1 cm

$$\text{Hence } C = \frac{A_{340\ nm}/\text{min.}}{6.2 \times 10^3 \times 1} \text{ (moles/litre)min-1}$$

$$C = \frac{A_{340\ nm}/min.}{6.2} \quad (\mu moles/ml)min\text{-}1$$

Specific activity = μ moles product formed $min^{-1}mg^{-1}$ protein

$$= \frac{A_{340\ nm}/min.}{6.2 \times mg\ enzyme/ml\ reaction\ mixture}$$

Since in these dehydrogenases (DHFR, LDH and MDH) the correspondence between coenzymes oxidised and product formed is 1:1. Therefore in the present study specific enzyme activity has been expressed as moles of NAD^+ or $NADP^+$ formed $min^{-1}mg^{-1}$ protein.

II.3 RESULTS AND DISCUSSION

Reverse micellar medium was found to be a versatile medium for *in vitro* study of enzymes DHFR, LDH and MDH. Water solubilized in the polar core of the reverse micelles forms water pool where entrapped enzymes are protected from the unfavourable action of organic solvents and surfactant molecules. In this way the reverse micelles behave as novel microreactors which accommodate enzymes and all the substrates in the water pool.

II.3.1 Solubilization of enzymes via reverse micelles

The solubility of these enzymes in CTAB reverse micellar system was found to be dependent on surfactant concentration, the

molar ratio of water to surfactant (w_o),type and concentration of buffers, concentration of aqueous stock solution of enzymes, temperature etc. Reverse micellar solutions of these water soluble enzymes in CTAB/isooctane:chloroform (1:1,v/v) were found to be homogeneous and optically transparent under specific conditions. This indicates that these enzymes in reverse micelles are solubilized according to water-shell model by which the enzymes reside in the centre of the water pool, surrounded by shell of water molecules which protects the enzymes from the surfactant wall and from the bulk organic solvents. It is expected that only a small fraction of the micelles are filled by enzyme molecules, the rest being available for coenzyme, substrate and water molecules. These enzyme containing micelles, coenzyme and substrate containing micelles, the micelles containing enzyme-substrate complex, and the unfilled micelles are considered to be in rapid equilibrium i.e. exchanging guest molecules very rapidly.

Concentration of solubilized enzyme or substrate in the reverse micelle can be expressed in two different ways. One is relative to the water pool, where enzyme reaction takes place and the other is relative to the overall system. For a compound which is practically insoluble in the organic solvent, the two concentration are as follows.[17]

$$C_{ov} = C_{wp}F_w$$

where C_{ov} and C_{wp} are the overall concentration and the water pool concentration respectively and F_w is the water volume

fraction which is given as

$$F_w = W_o[CTAB](1.8)/100$$

In the calculations of present study the overall concentration has been considered throughout unless stated otherwise. The concentration of aqueous stock solution of enzyme and salt concentration in which enzyme is stored as a suspension, was found to affect the solubility of enzymes in reverse micelles very significantly. DHFR which is a small enzyme (MW $\approx$ 20000 dalton)[5,18] was not solubilized easily as compared to the MDH (MW $\approx$ 70000 dalton and two subunits of 35000 dalton each)[12,18,19] and to the large and complex enzyme lactate dehydrogenase (MW $\approx$ 140,000 dalton comprising of four subunits).[6,18] The main reason for this was that for the aqueous stock solution of enzyme to be entrapped in reverse micelles, DHFR was diluted upto about 20 fold from its commercially supplied stock solution whereas LDH and MDH were diluted upto about 250 to 500 times. These dilutions were made to adjust the initial velocity of enzyme reaction in a better measurable range. At these dilutions the concentration of aqueous stock solution of DHFR to be solubilized in reverse micelles, was about 0.1 mg/ml whereas for LDH or MDH, it was about 0.01 mg/ml. On the other hand the concentration of ammonium sulfate (these enzymes are stored as suspension in about 2-4 M ammonium sulfate) after the enzyme dilution was about 200 mM for DHFR and about 10 mM for LDH or MDH. Higher salt concentrations in the case of DHFR created solubilization problems because only suitable dilute solutions can penetrate the wall of the micellar core. Type and concentration of buffer, in

which aqueous stock solutions were prepared, were also found to affect the solubility of enzymes in reverse micelles. A buffer in which assay is carried out in aqueous medium may or may not be suitable for the assay conditions in reverse micelles.

II.3.2 Enzyme activity in reverse micellar solution

Reverse micelles have been found to prevent enzyme denaturation by organic solvents and surfactant molecules. In the reverse micelles the molar ratio of water to surfactant (w_o = $[H_2O]/[CTAB]$) is an important parameter which defines the size and properties of the water pool.[20-22] The specific activity of enzymes was found to be dependent on various factors like w_o, pH, surfactant concentration etc.

II.3.2.1 Influence of degree of hydration on enzyme activity

One of the most striking effect observed in the study of behavior of enzymes in reverse micelles is the dependence of catalytic activity of solubilized enzymes on the degree of hydration (w_o) of the reverse micelles.

The effect of water content of DHFR activity (Fig. II.1) shows that the catalytic efficiency of the enzyme increases with increasing w_o reaching a maximum at 13.33, this value of w_o is called the optimum w_o ($w_{o,opt.}$). Beyond this w_o values decrease in activity occurs. At a value of w_o 13.33, pH 7.0, CTAB 75 mM the enzyme activity was higher than that in aqueous buffer which is referred to as "super activity". Fig. II.2 shows the effect of

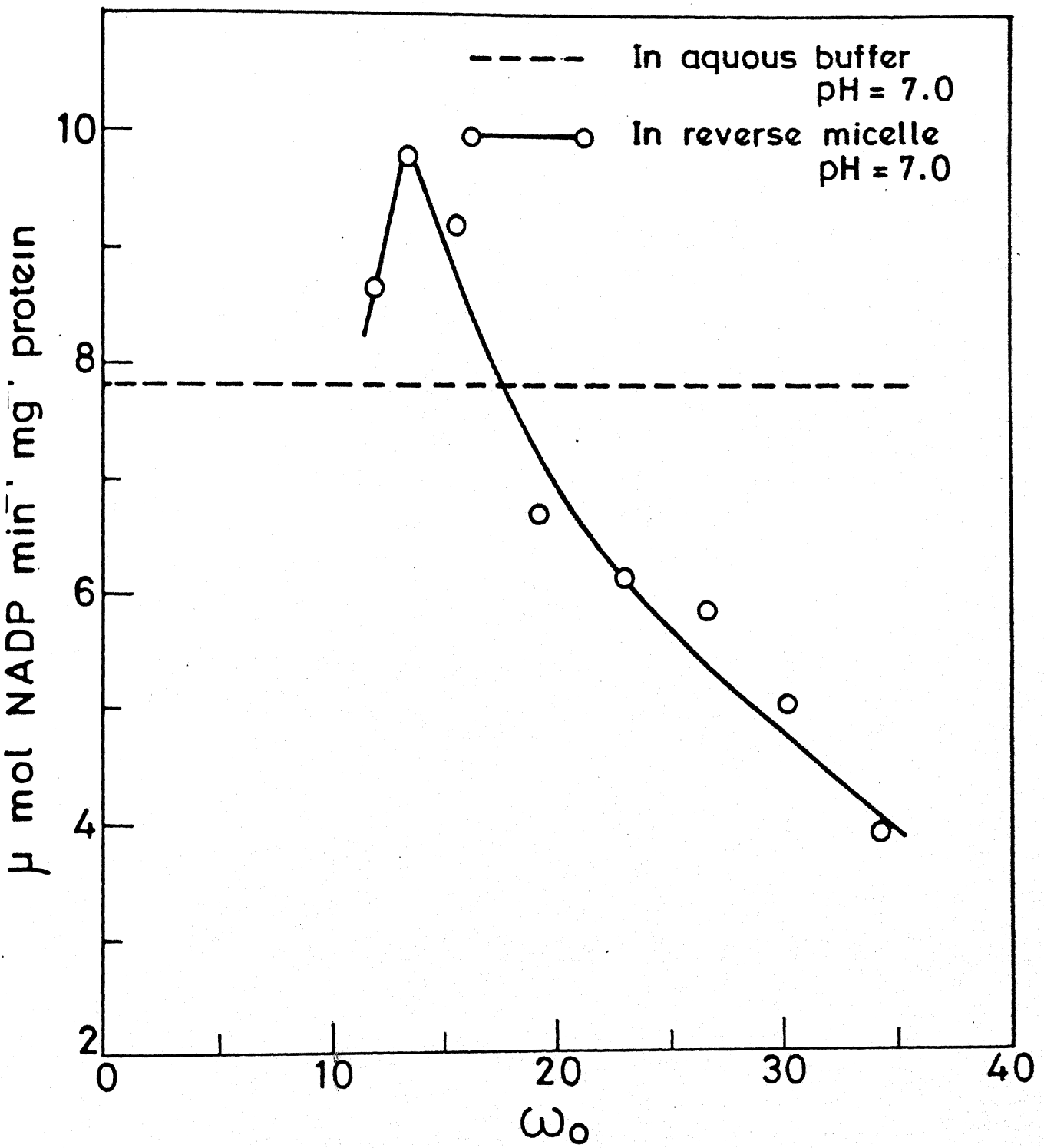


Fig.II.1. Specific activity of DHFR in CTAB-isooctane-chloroform-water reverse micelles as a function of w_o. The concentrations were [CTAB]=100 mM; [$NADPH^o$]=0.06 mM; [FAH_2]=0.06 mM and [β-mercaptoethanol]=2 mM. Buffer was 25 mM Tris-HCl, pH = 7.0.

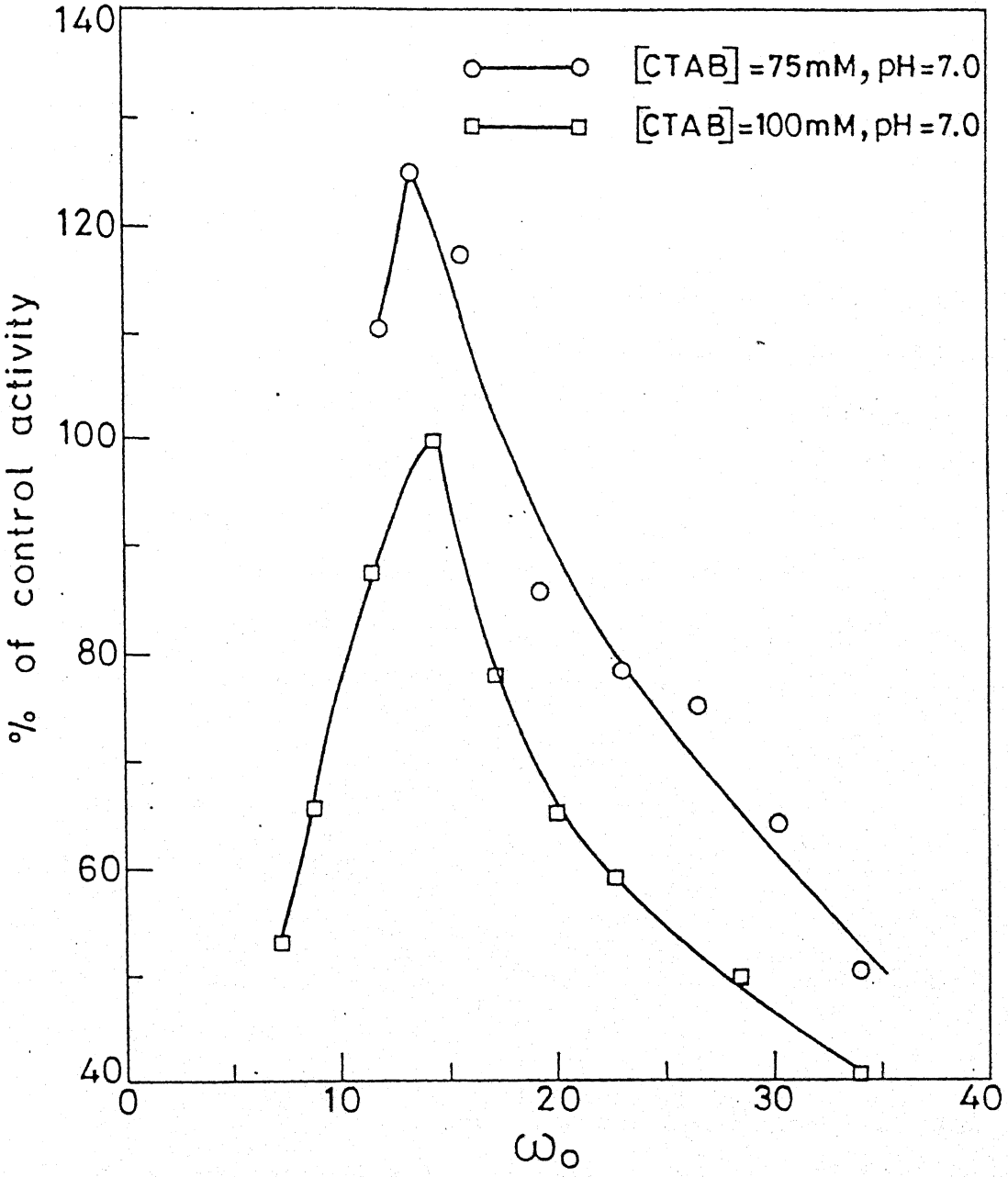


Fig.II.2. DHFR activity vs w_o profiles at two CTAB concentrations: 75 mM CTAB (O—O) and 100 mM CTAB (□—□). The activity is expressed relative to the activity in aqueous buffer. Other concentrations were same as in Fig.II.1.

degree of hydration on percentage control activity of DHFR at two different surfactant concentrations namely 100 mM and 75 mM CTAB. In these plots optimum values of w_o are different. At 75 mM CTAB, optimum w_o was 13.33 with corresponding enzyme activity being 125% of the value found in aqueous buffer pH 7.0 whereas for 100 mM CTAB the value of optimum w_o was 14.44 which corresponds to the full enzyme activity as compare to the aqueous medium. Below and above of these optimum w_o values a decrease in enzyme activity was found. Enzyme activity w_o profile as a function of pH is shown in Fig. II.3. For each pH_{stock} (pH of the buffer solution injected into the reverse micellar solution), a bell-shaped curve is obtained. In the present study the term pH has been used in place of pH_{stock}. This pH_{stock} may or may not be the actual pH inside the core of reverse micelles. These type of bell shaped curves have also been found for other enzymes. For example, α-chymotrypsin,[17] horse liver alcohol dehydrogenase,[23] yeast alcohol dehydrogenase,[27] lysozyme,[25] glutathione reductase,[24] lactate dehydrogenase,[26] malate dehydrogenase[16] etc. From these cases, it appears that the bell-shaped dependence of enzyme activity upon w_o represents a general feature of the micellar enzyme solution.

Effect of w_o on LDH activity has been shown in Fig. II.4. At initial w_o enzyme activity was almost equal to zero. An increase in water pool size showed increase in enzyme activi touching a maximum at w_o 30.55. This highest activity in reverse micelles was equal to the activity observed in aqueous buffer at optimum conditions. Variations of enzyme activity with degree of

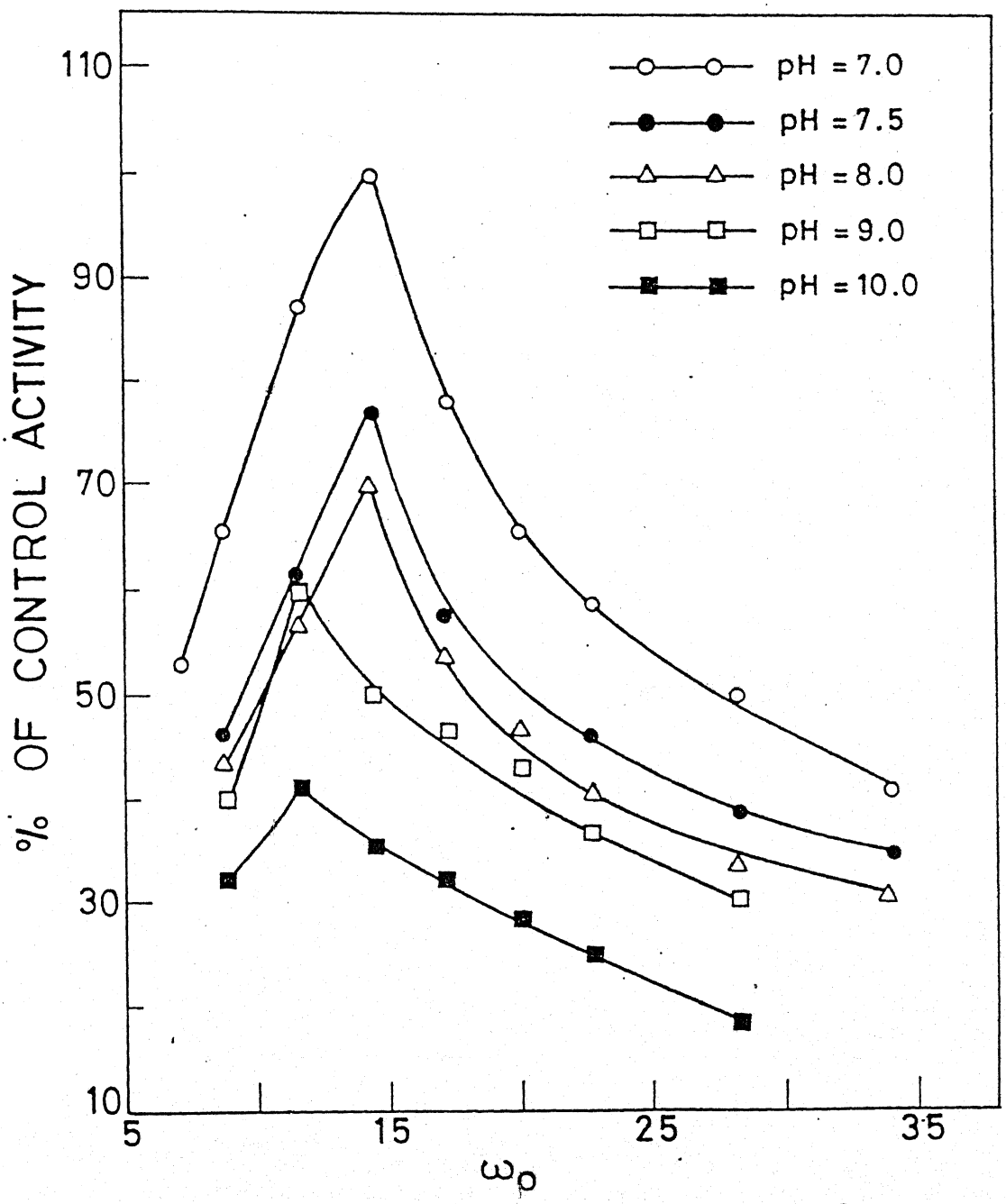


Fig.II.3. Percentage control activity of DHFR plotted as a function of w_o at different pH values. Buffers were 25 mM tris-HCl (pH=7-8) and 25 mM glycine-KOH (pH=9-10). Surfactant, substrate, coenzyme concentrations were the same as in Fig.II.1.

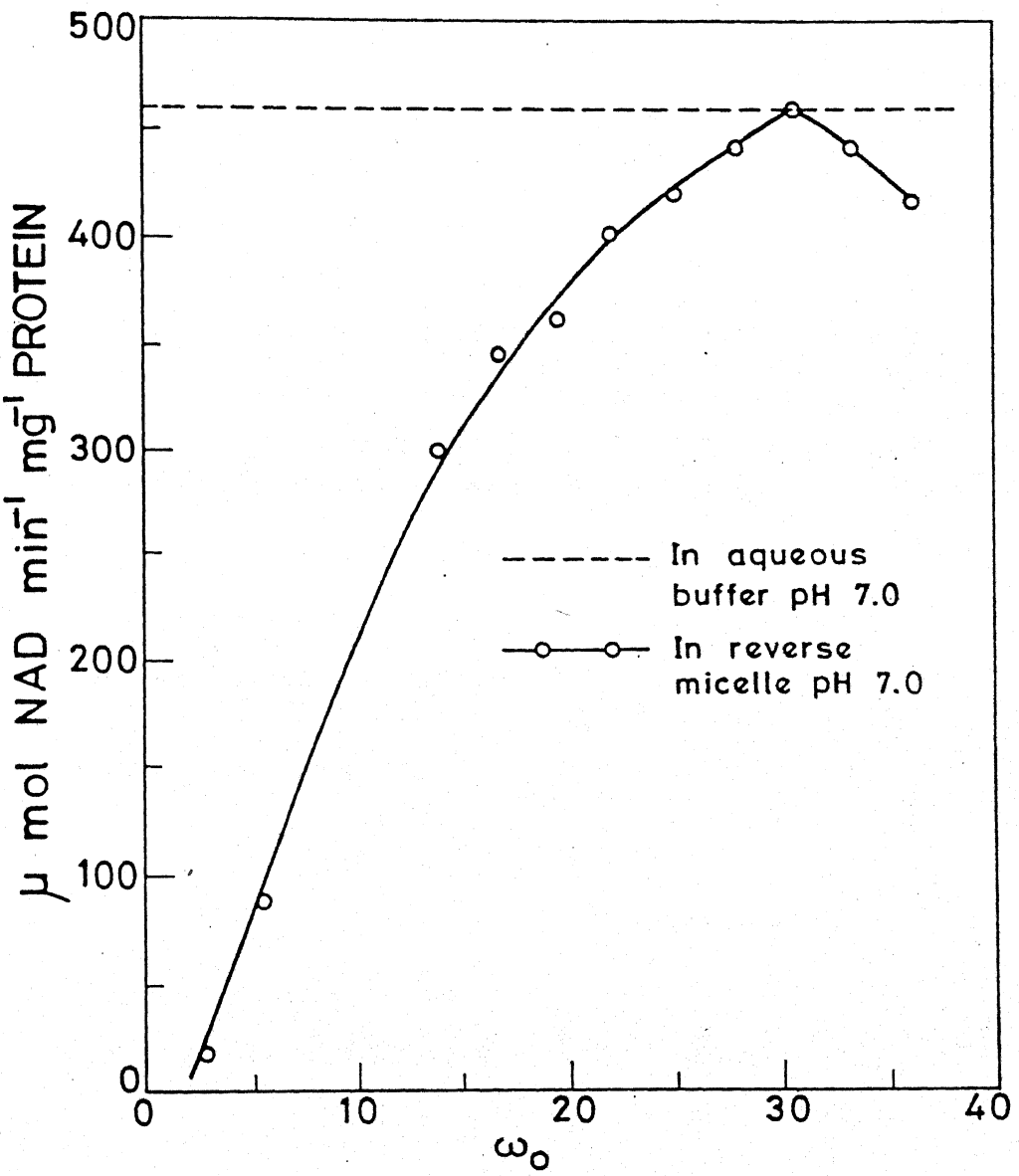


Fig.II.4. Specific activity of LDH as a function of w_o in CTAB reverse micelles in isooctane:chloroform (1:1, v/v) water mixture. The concentrations were as follows: [CTAB] = 0.1M; [NADH] = 0.2 mM; [Sodium pyruvate] = 1 mM; and Potassium phosphate buffer, 0.1M, pH 7.0.

hydration (w_o) at different pH values are shown in Fig. II.5. For every pH value almost same shape of curve has been obtained in which enzyme activity first increases with increasing w_o and reaching a maximum at particular w_o, later it shows decrease in enzyme activity with further increase in w_o values. Enzyme activity maximum has been found to decrease with increasing pH values. The data on w_o dependence of enzyme activity indicate that size of the water pools of the reverse micelles plays a very important role in regulating the enzyme activity.

Figure II.6 gives the variation of specific activity of MDH with respect to the degree of hydration at pH 7.5 (equal to the optimum pH in aqueous buffer) in micellar media. Upto the w_o value 5, the enzyme activity was very low and the maximum enzyme activity was obtained at w_o value 33.33. The variation of specific activity of MDH with w_o values at pH 10.3 has been shown in Fig. II.7. Similar to the data of Fig. II.6, enzyme activity at initial w_o values was low but later increased with increasing w_o. The maximum enzyme activity in this case was at the w_o value 25.55. Fig. II.8 gives the data on the variation of initial rate of enzyme MDH as a function of w_o at different pH values. It may be noted that the enzyme activity increases with increasing values of w_o, the lowest activity values being at the starting w_o value of 3.33. Upto the w_o value of 6, the enzyme activity was ranging between 5-20% of the activity found in aqueous buffer. Similar to the behavior of other enzymes each activity - w_o profile is a bell-shaped curve. The maxima on the curve determines the value of w_o at which the enzyme shows maximum

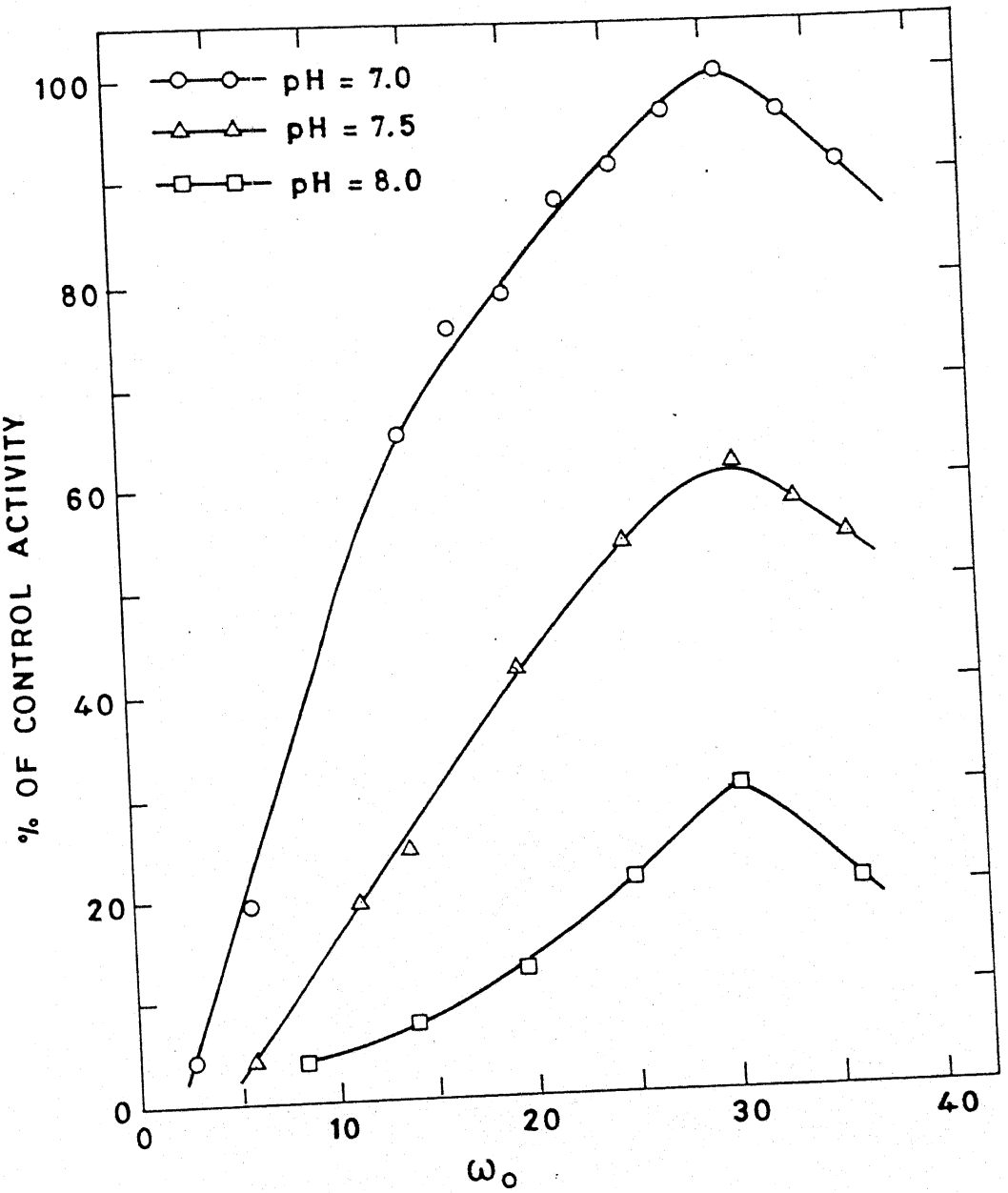


Fig.II.5. LDH activity–w_o profiles as a function of pH in CTAB reverse micellar system. Substrate, coenzyme and buffer concentrations were the same as in Fig.II.4. The enzyme activity expressed is relative to the activity in aqueous buffer (0.1M potassium phosphate, pH 7.0).

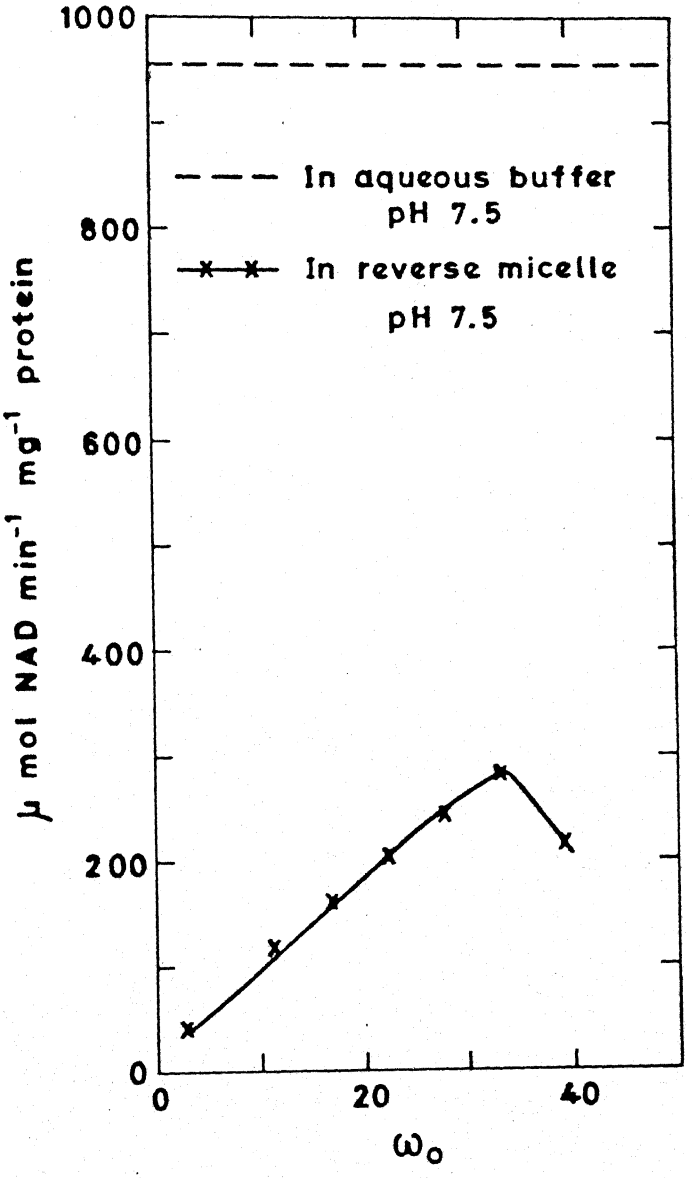


Fig.II.6. Dependence of the specific activity of MDH on the w_o in the micellar system of CTAB/chloroform:iso-octane (1:1, v/v). Concentrations were: [CTAB] = 0.1M; [NADH] = 0.2 mM; [Oxaloacetate] = 0.5 mM; The potassium phosphate buffer 0.1M, pH 7.5) was same in both aqueous and reverse micellar system.

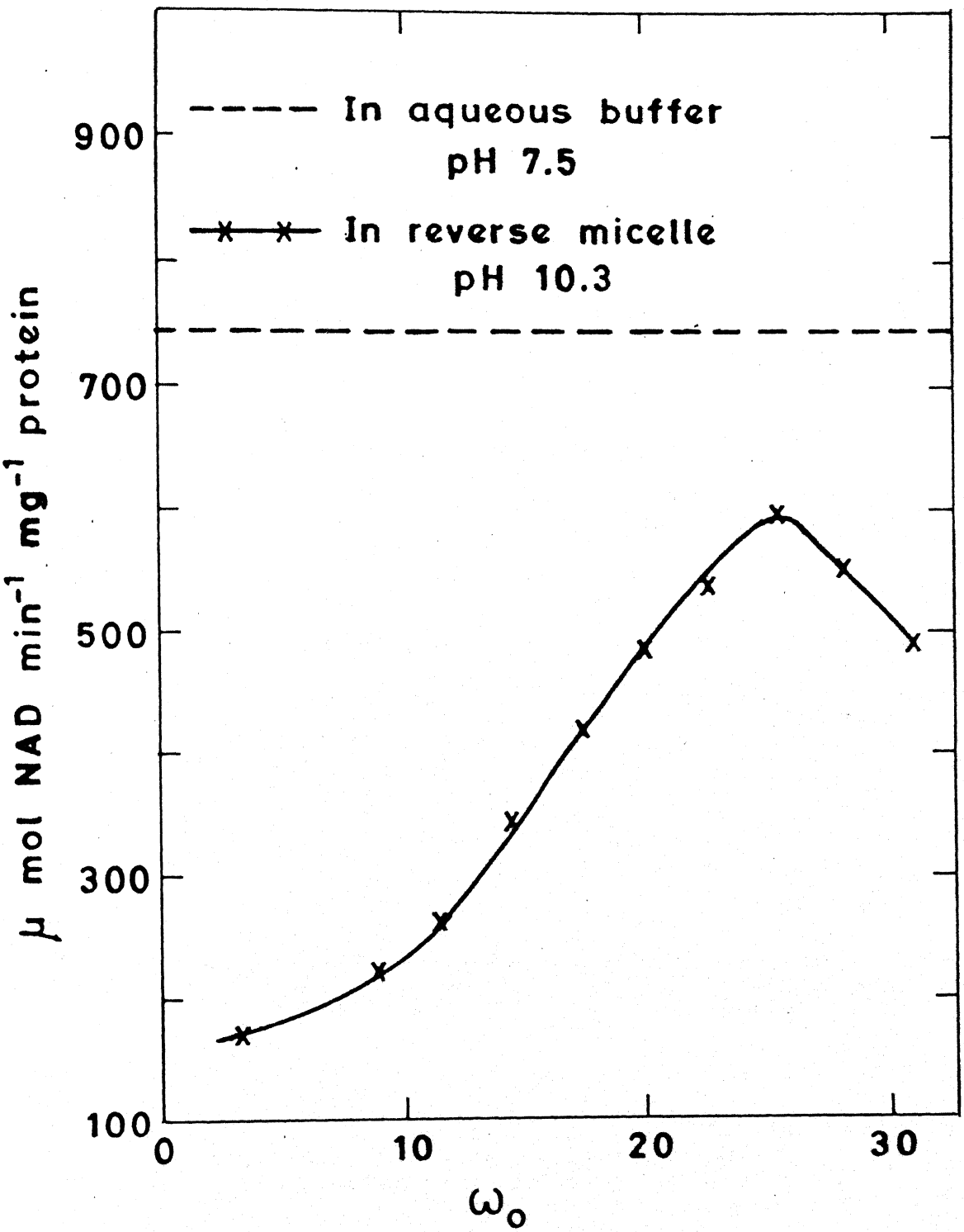


Fig.II.7. Effect of w_o on the specific activity of MDH at pH 10.3 in CTAB reverse micellar system. CTAB, NADH and oxaloacetate concentrations were the same as in Fig.II.6. The buffers were: 0.1Mpotassium phosphate, pH 7.5 (in aqueous system) and 0.1Mglycine KOH, pH 10.3 (in reverse micelles).

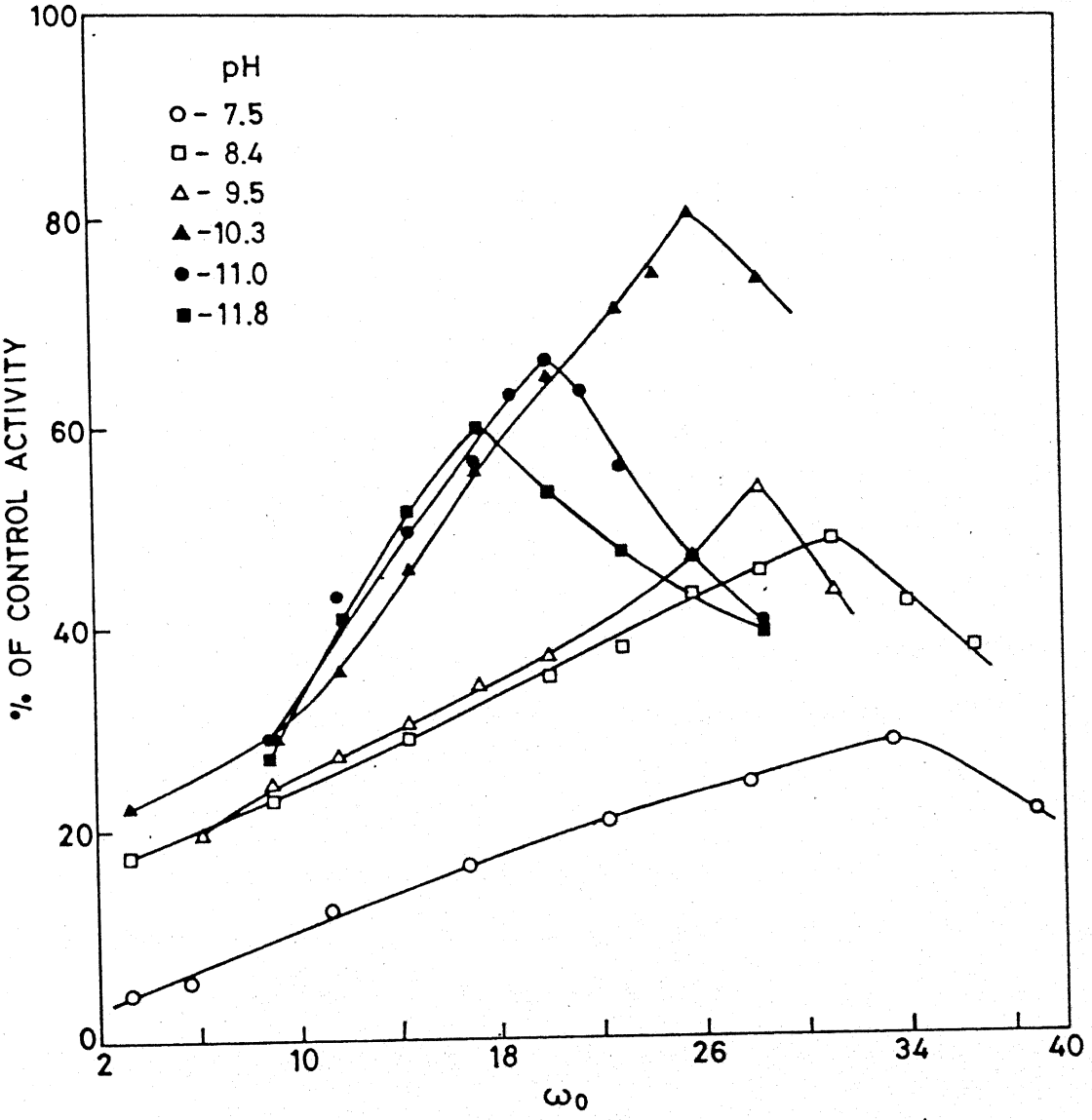


Fig.II.8. Variation of percentage control activity of MDH with w_o at fixed values of pH in CTAB/chloroform:isooctane (1:1, v/v). Buffers were 0.1Mpotassium phosphate (pH 7-8.5) and 0.1Mglycine-KOH (pH 9-12). Other concentration were the same as in Fig.II.6.

activity. The maximum enzyme activity was achieved at $w_o = 25.55$ and pH 10.3. This value is almost equal to that found in aqueous medium at pH 7.5. Fig. II.9 shows the percentage control activity variation with optimum w_o obtained at different pH. Optimum w_o for MDH was in the range of 15-35 which corresponds to the % control activity in the range of 20-80%. Maximum percentage activity was at optimum $w_o = 25.55$.

II.3.2.2 Influence of pH on enzyme activity

The rate of enzyme reactions and the kinetic parameters characterising them in most of the cases depend upon the pH of the buffer solution. When an enzyme reaction is transferred from an aqueous solution to reverse micellar system, significant changes in pH might occur.

The pH dependence of dihydrofolate reductase activity in reverse micellar solution at different w_o values is shown in Fig. II.10. Important feature of the pH data for all w_o values in the loss of enzyme activity at pH values lower and greater than 7.0 which is similar to the effect of pH in aqueous medium. With few exception, this kind of pH profile in reverse micelles is an unusual phenomenon and has not been observed for more enzymes studied in reverse micelles. In general it is found that pH profile for enzymes in reverse micelles shifts to the alkaline side.[16,17,23,27]

Variation of max % control activity and optimum w_o with pH for DHFR are shown in Fig. II.11. Fig. indicates that max.

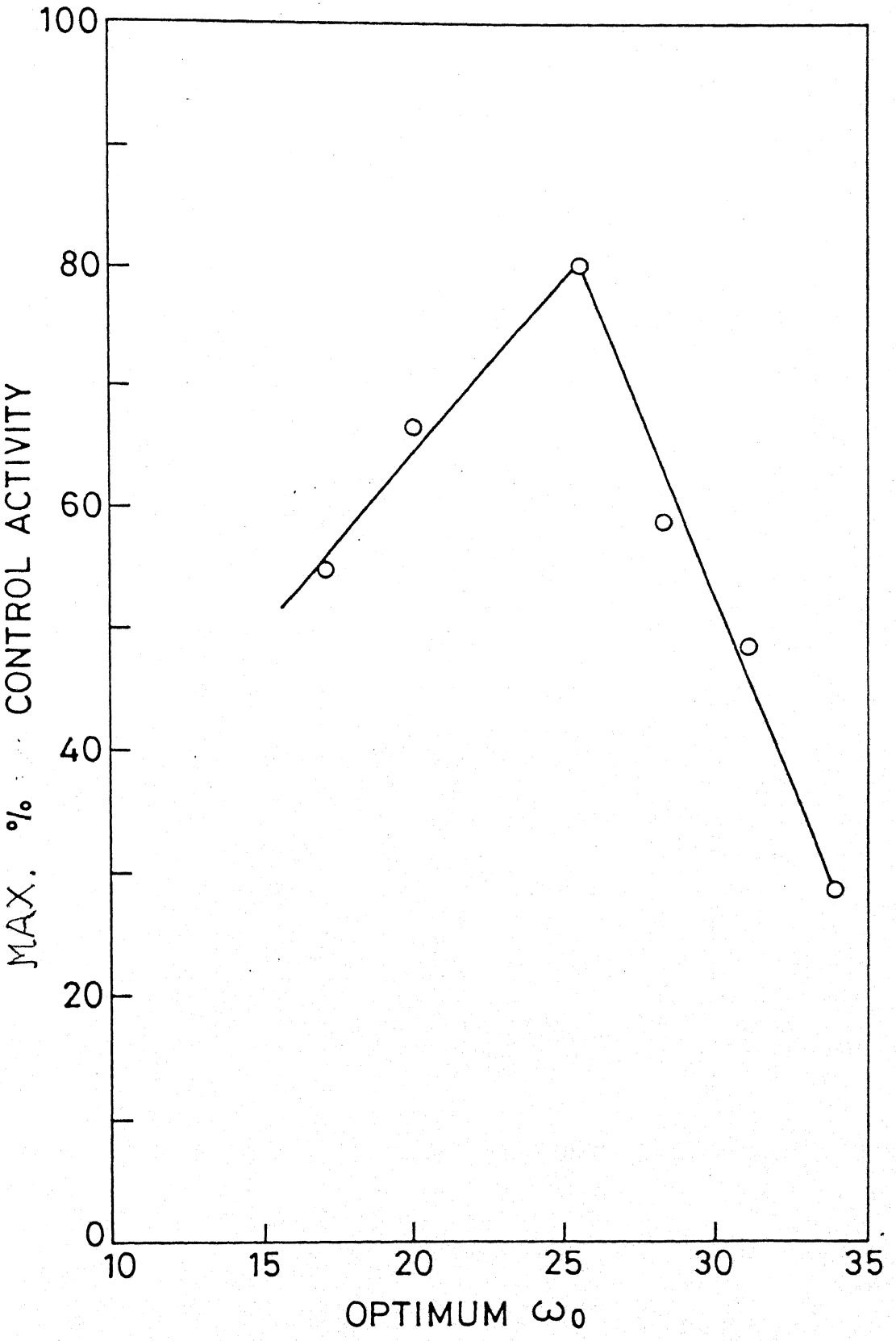


Fig.II.9. Variation of maximum percentage control activity of MDH with optimum w_o in CTAB micellar system.

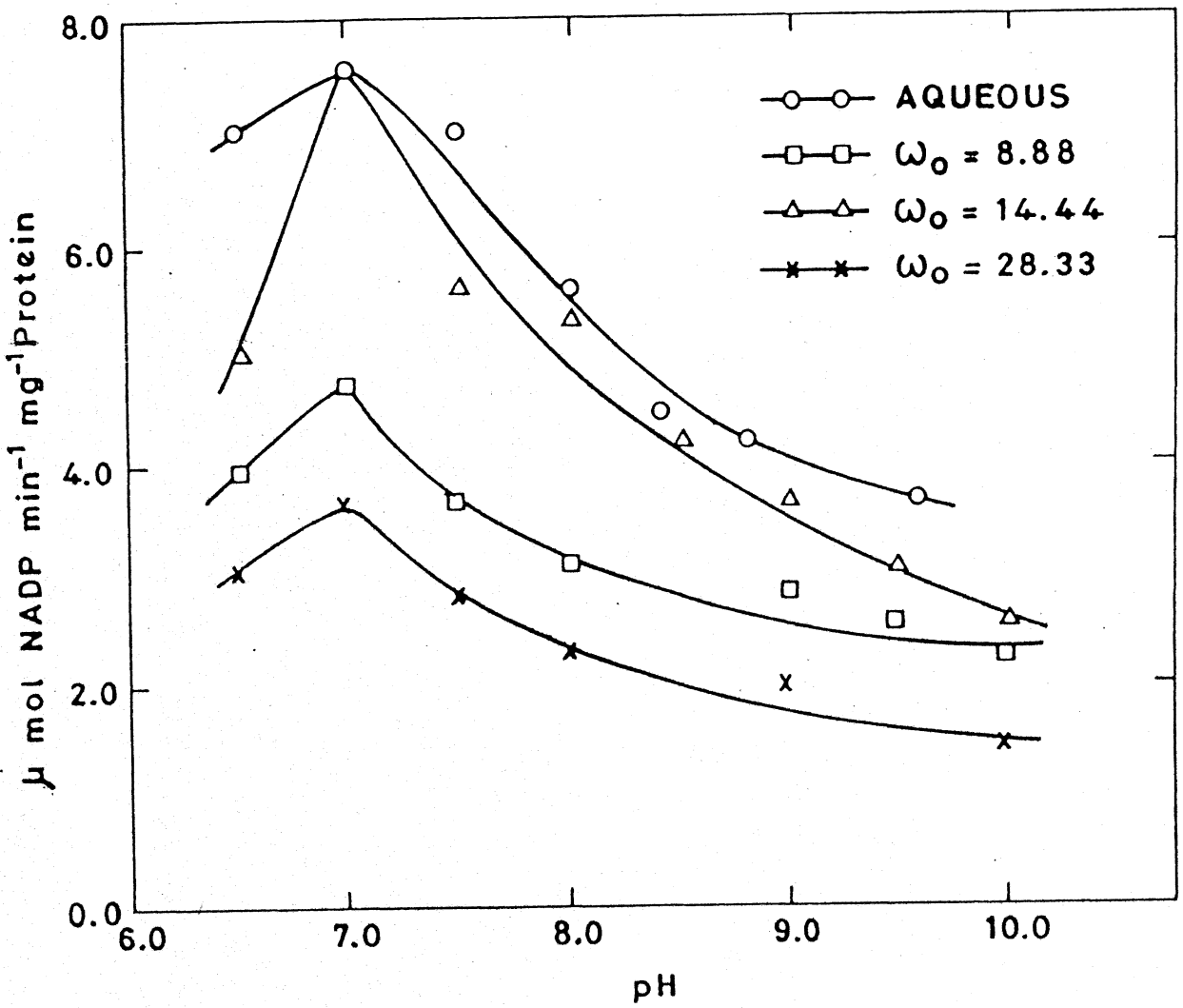


Fig.II.10. Dependence of DHFR activity on the pH of the bulk water (buffer) transferred to the micellar solution of CTAB/isooctane:chloroform (1:1, v/v) at different w_o values.

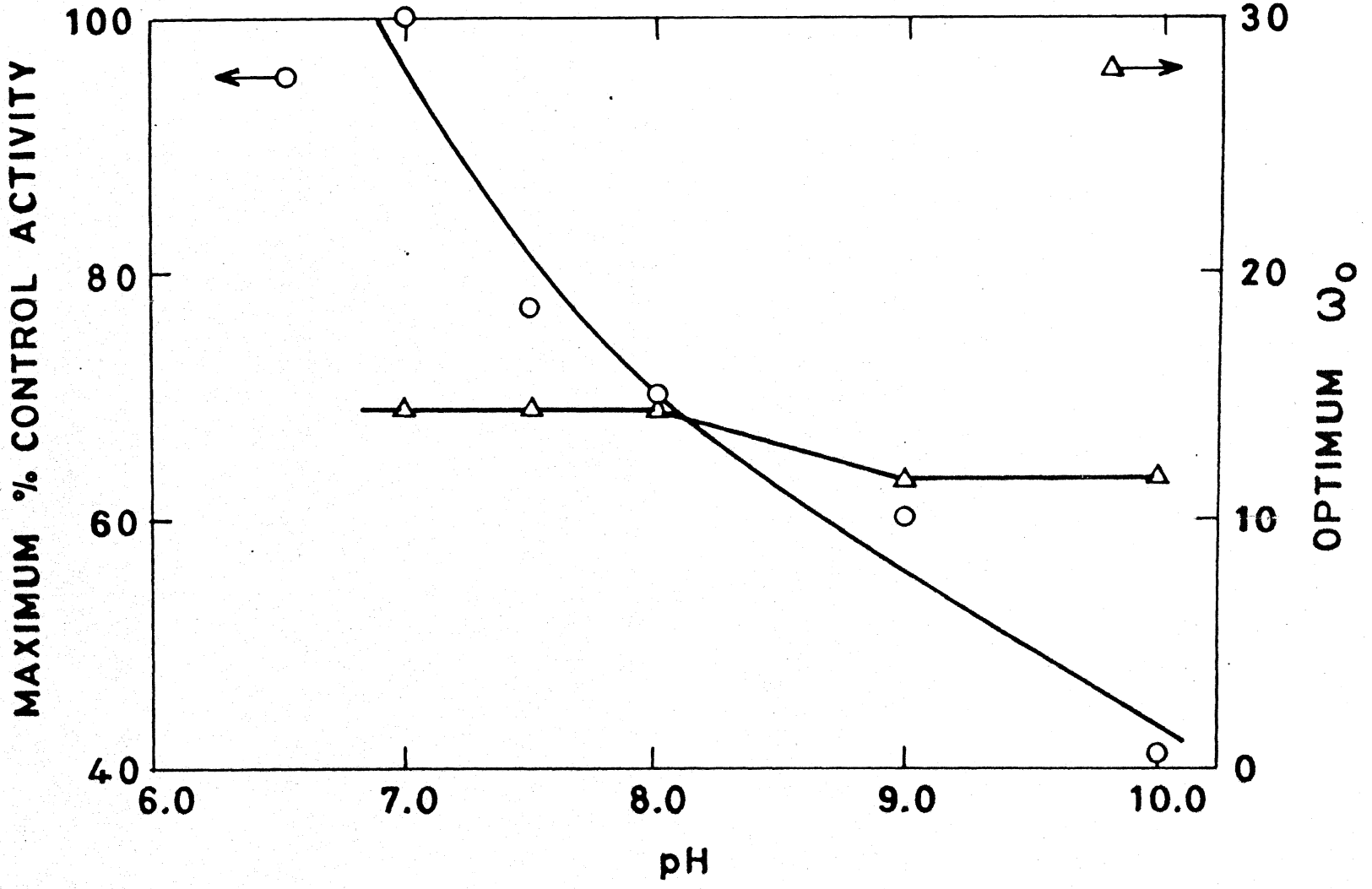


Fig.II.11. Variation of maximum percentage control activity (O-O) and optimum w_o (Δ-Δ) with the pH for DHFR in CTAB micellar system.

enzyme activity decreases almost linearly with increasing pH whereas optimum w_o shows very small change from pH range 7.0-10.0. For pH range 7-8, optimum w_o was constant where as for pH range 8-9 the optimum w_o showed slight decrease and again it was constant for pH range 9-10.

Specific enzyme activity - pH profile for LDH in both aqueous and reverse micellar media is shown in Fig. II.12. In both media the specific activity of LDH increases with the increase in pH, reaching a maximum at 7.0 and then starts decreasing. The effect of pH on enzyme LDH in reverse micellar media is similar to that of DHFR but different from other enzymes. In some enzymes the pH maximum in reverse micelles becomes 1-3 pH unit higher that the pH maxima in aqueous medium,[16,17,23,24,27] but in the case of LDH the optimum pH in both the media remains the same. Fig. II.13 shows the variation of enzyme activity of LDH as well as optimum w_o with pH. Maximum enzyme activity was at pH 7.0 which decreases linearly with increasing pH. Optimum w_o remained constant at 30.55 throughout the pH change in reverse micelles. At this w_o, an increase in pH by one unit shows 70% loss of enzyme activity.

Fig. II.14 gives the effect of pH on MDH activity in aqueous and reverse micelles at different w_o values. Activity of enzyme shows a maximum value at a particular pH. Above and below this pH, activity of enzyme decreases. It is interesting to note that in aqueous and reverse micelles at w_o higher than 20, the decrease in enzyme activity from its maximum limit is steep and

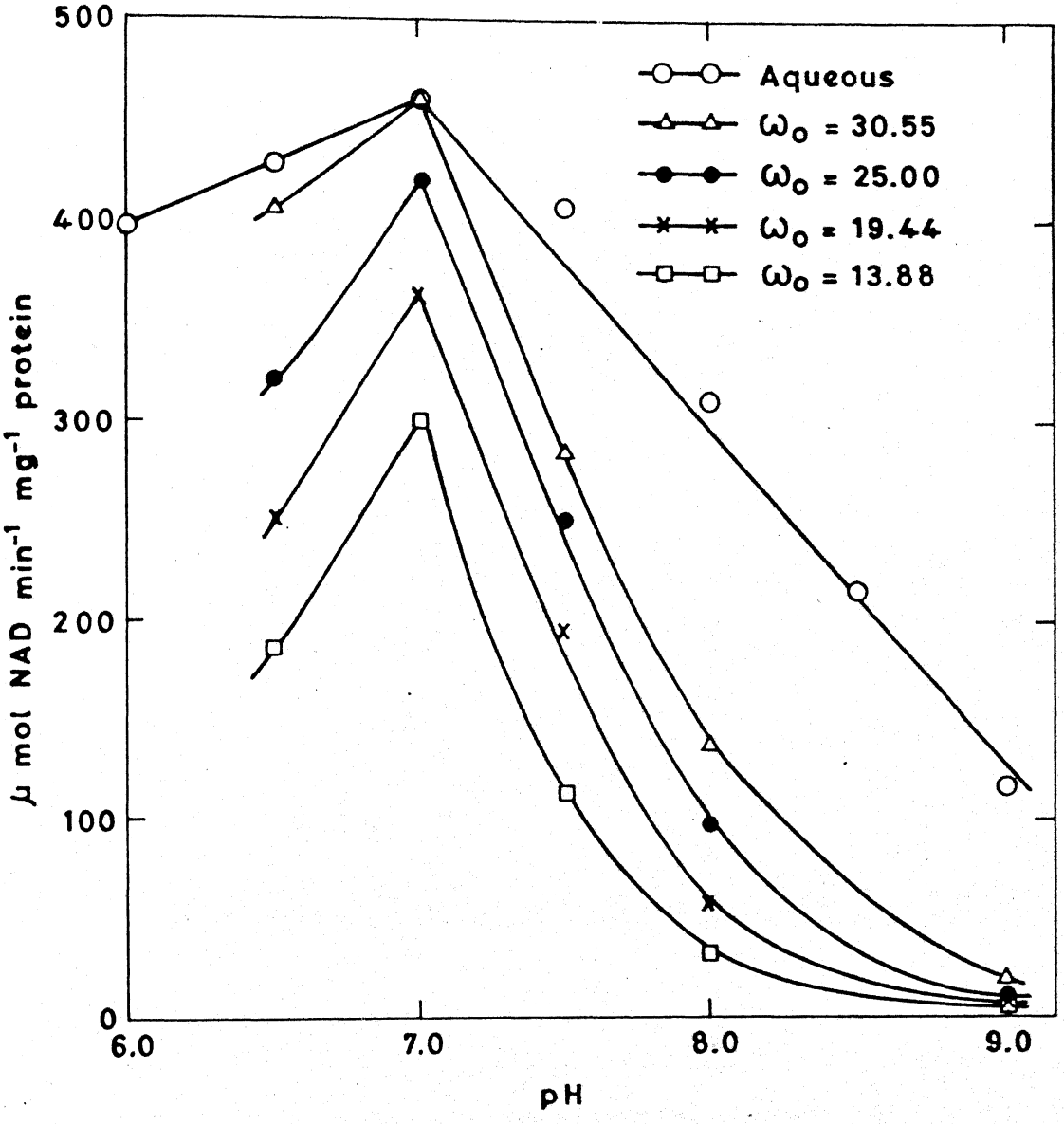


Fig.II.12. Effect of pH on LDH activity at different w_o values in CTAB micellar system.

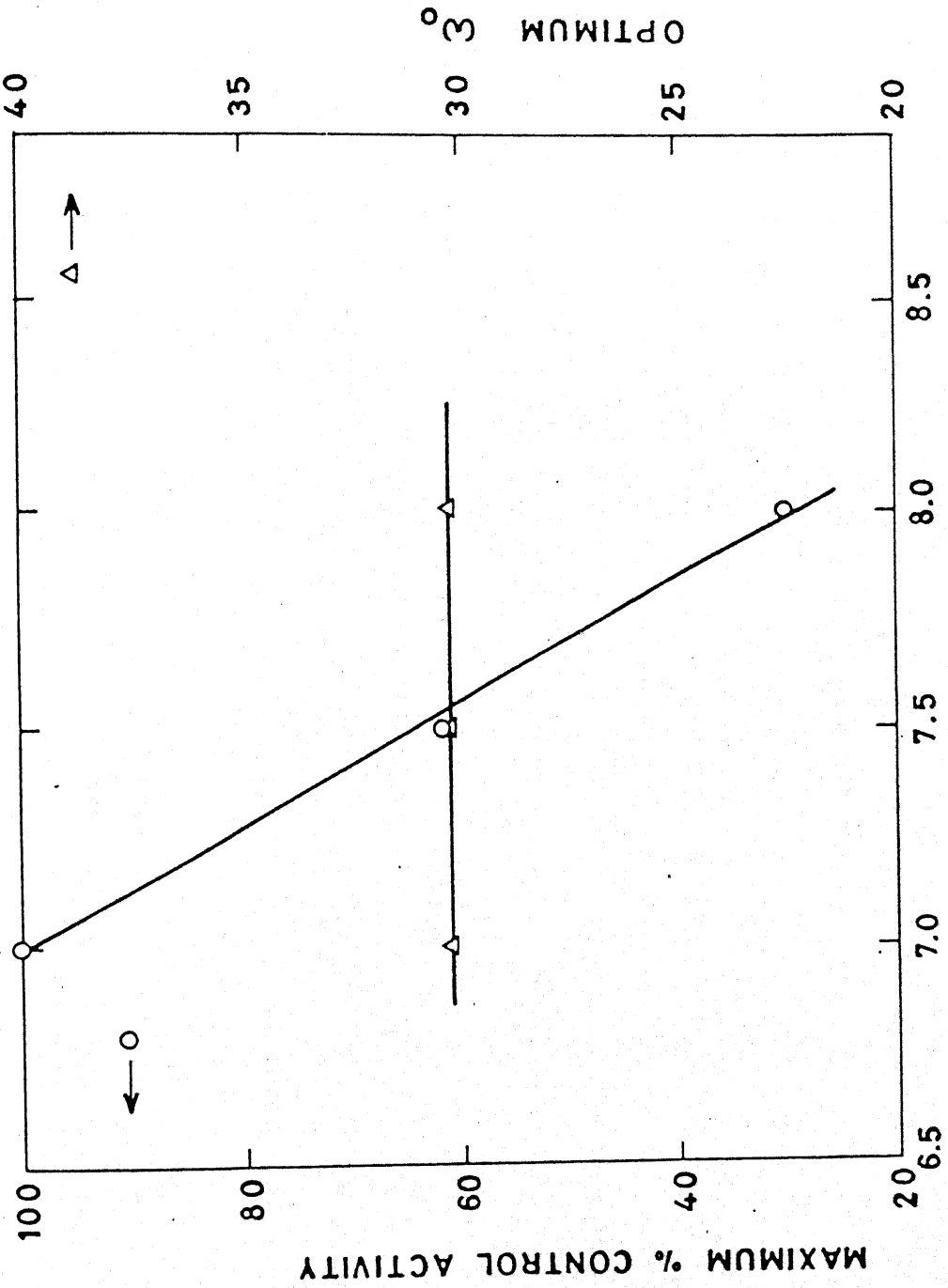


Fig.II.13. Variation of maximum LDH activity (O-O) and optimum w_o (Δ-Δ) with the pH in CTAB/isooctane-chloroform (1:1, v/v).

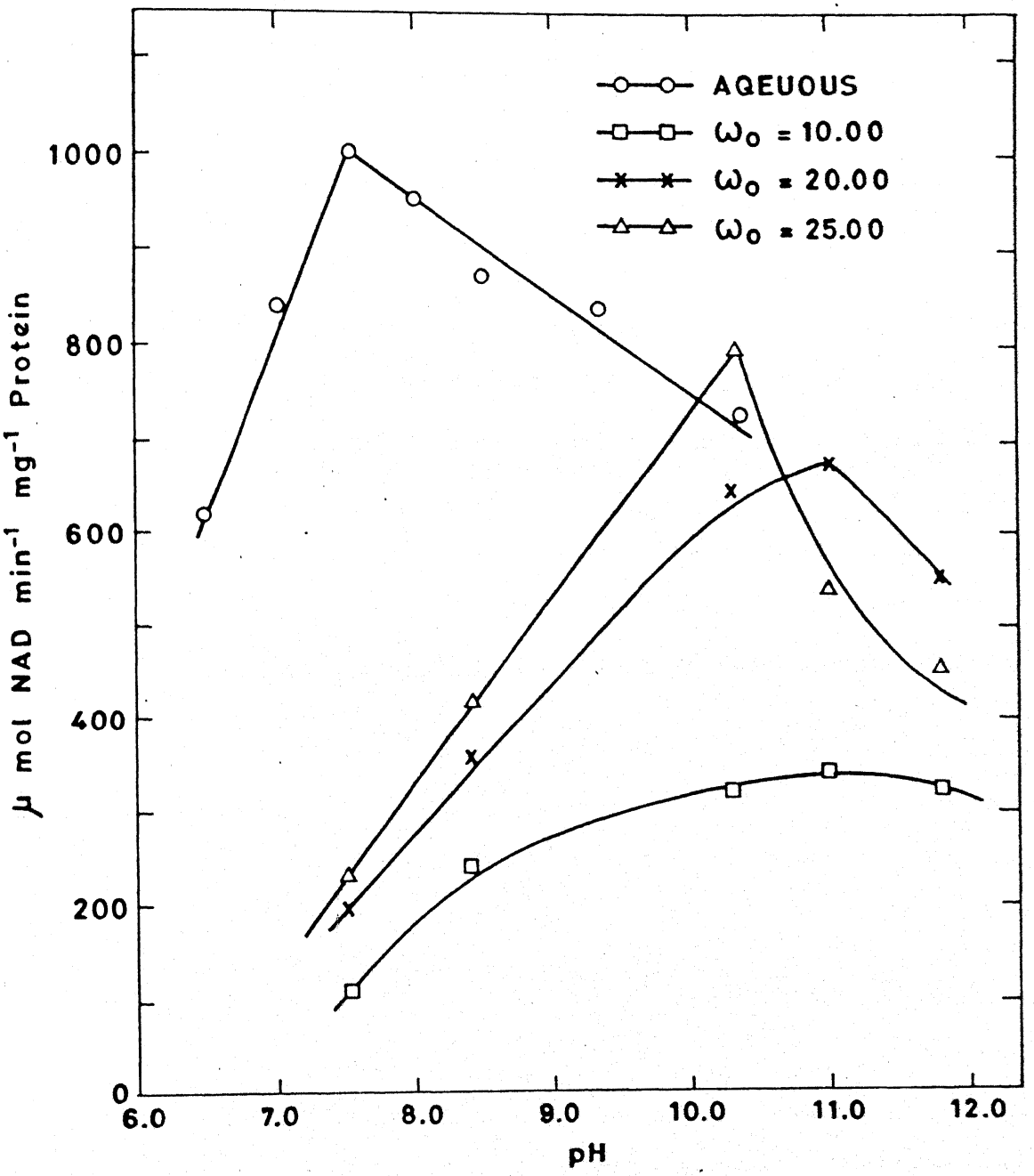


Fig.II.14. Specific activity vs pH profiles of MDH at different w_o values in CTAB reverse micellar system.

almost linear whereas at lower w_o, the decrease is less steep and in the form of a curve. In aqueous solution the enzyme MDH exhibits maximum activity at pH 7.5, whereas in reverse micelles the maximum activity occurs at pH 10.3. This type of pH shift has been observed for other enzymes in reverse micelles. Unlike DHFR and LDH, the maximum enzyme activity-pH profile for MDH is bell shaped Fig. II.15(a) with a maximum at pH 10.3. At this maximum pH (10.3) and w_o 25.55 the enzyme activity is 80% of the activity in aqueous buffer at its optimum conditions. Fig. II.15(b) shows the variation of pH as a function of optimum w_o ($w_{o\ opt.}$). It is found that by increasing pH of the buffer solution the optimum w_o decreases in almost linear fashion.

II.3.2.3 Influence of surfactant concentration on enzyme activity

For the display of maximum enzyme activity in reverse micelles, optimum concentration of the surfactant is critical. Almost all the enzymes have different values for optimum surfactant concentration.

Variation of surfactant (CTAB) concentration for DHFR showed that enzyme activity was highly regulated by this parameter (Fig. II.16). At two optimum conditions (w_o = 14.44, pH 7.0 and w_o = 11.66, pH 9.0) the activity CTAB concentration profile displays maximum at 75 mM CTAB concentration. In the condition CTAB = 75 mM, w_o = 14.44, pH 7.0, the enzyme activity was 120% of the activity found in aqueous buffer at its optimum conditions whereas at CTAB = 75 mM, w_o = 11.66, pH 9.0, the enzyme activity was reduced to one half of the value found in the former case.

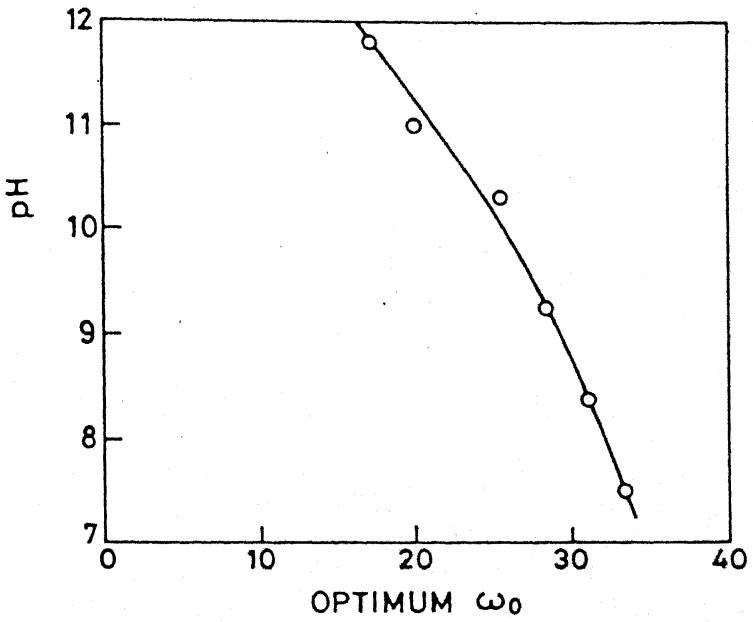


Fig.II.15(b)

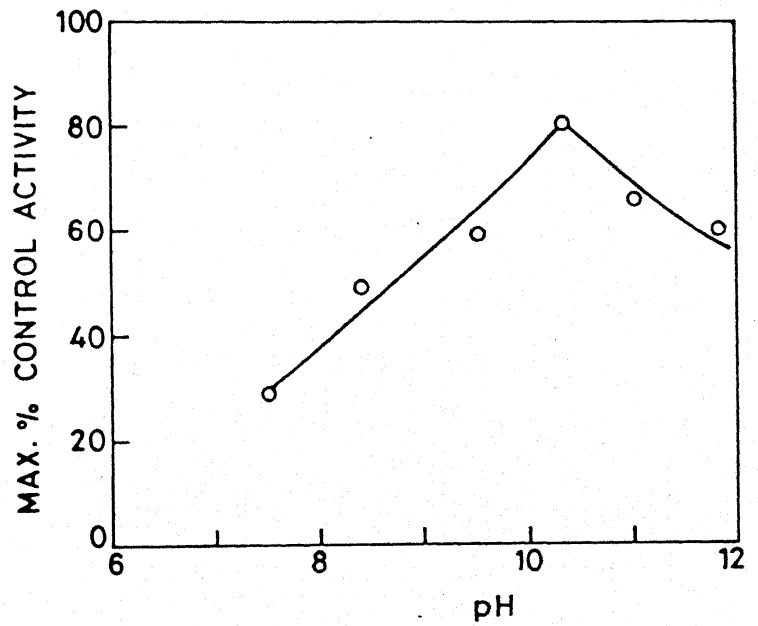


Fig.II.15(a)

Fig.II.15(a) Maximum MDH activity plotted as function of pH in reverse micellar system of CTAB in isooctane-chloroform.

(b) Variation of optimum pH with optimum w_o for MDH in CTAB/chloroform-isooctane (1:1, v/v) reverse micellar system.

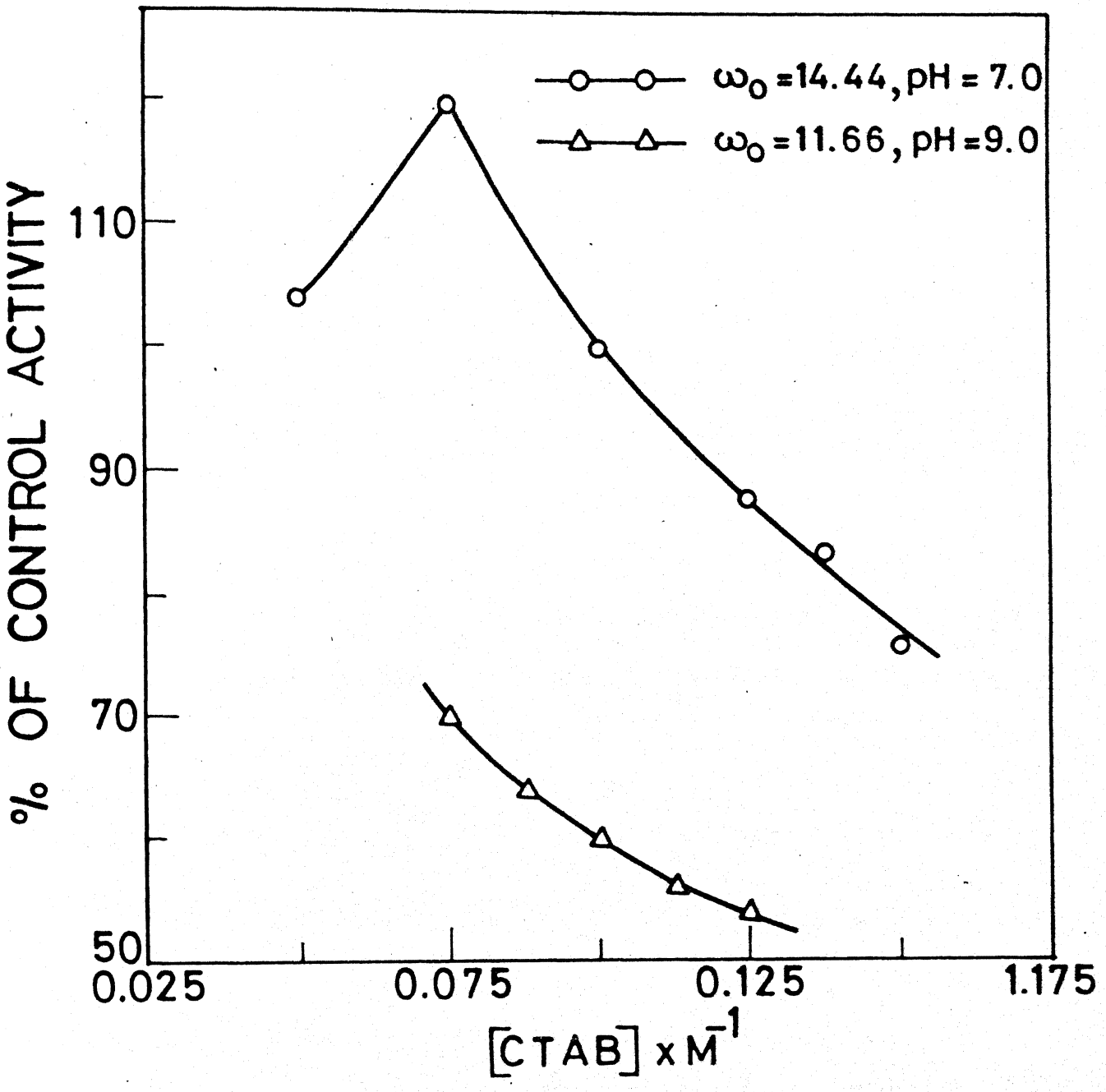


Fig.II.16. DHFR activity vs surfactant concentration profile at fixed pH and w_o values in CTAB micellar system.

The concentration of surfactant influences the enzyme activity of LDH remarkably as shown in Fig. II.17. The maximum enzyme activity was at CTAB concentration 0.1 M at both the w_o values viz. 30.55 and 15.55. Below and above this optimum CTAB concentration (0.1 M) the enzyme activity decreased. At the same CTAB concentration (0.1 M) lowering of w_o from 30.55 to 15.55 resulted in a loss of about 30% enzyme activity.

Percentage control activity - CTAB concentration profiles for MDH at w_o 25.55, pH 10.3 and w_o = 31.11, pH 8.4, are shown in Fig. II.18. The effect of surfactant concentration was similar to the case of LDH which displays maxima at CTAB concentration 0.1 M.

Water pool of the reverse micelles seems to be an important region. The size of the water pool controls the activity of enzymes entrapped therein. It seems that the optimum value of w_o (where enzyme activity is maximum) is different for different enzymes. The optimum value of w_o probability depends on the size and complexicity of the enzyme. Maximum enzyme activity of DHFR in CTAB reverse micellar system is not found at the largest possible water concentration, but rather at w_o values well below 15. Apparently the size of water pool inside the reverse micelle is such that it suitably accommodates the enzyme in its most active conformation. It is expected that, the maximum enzyme activity is found where the size of water pool resembles the size of enzyme. The super activity of the enzyme DHFR, is a rare phenomenon which may be partly attributed to the special

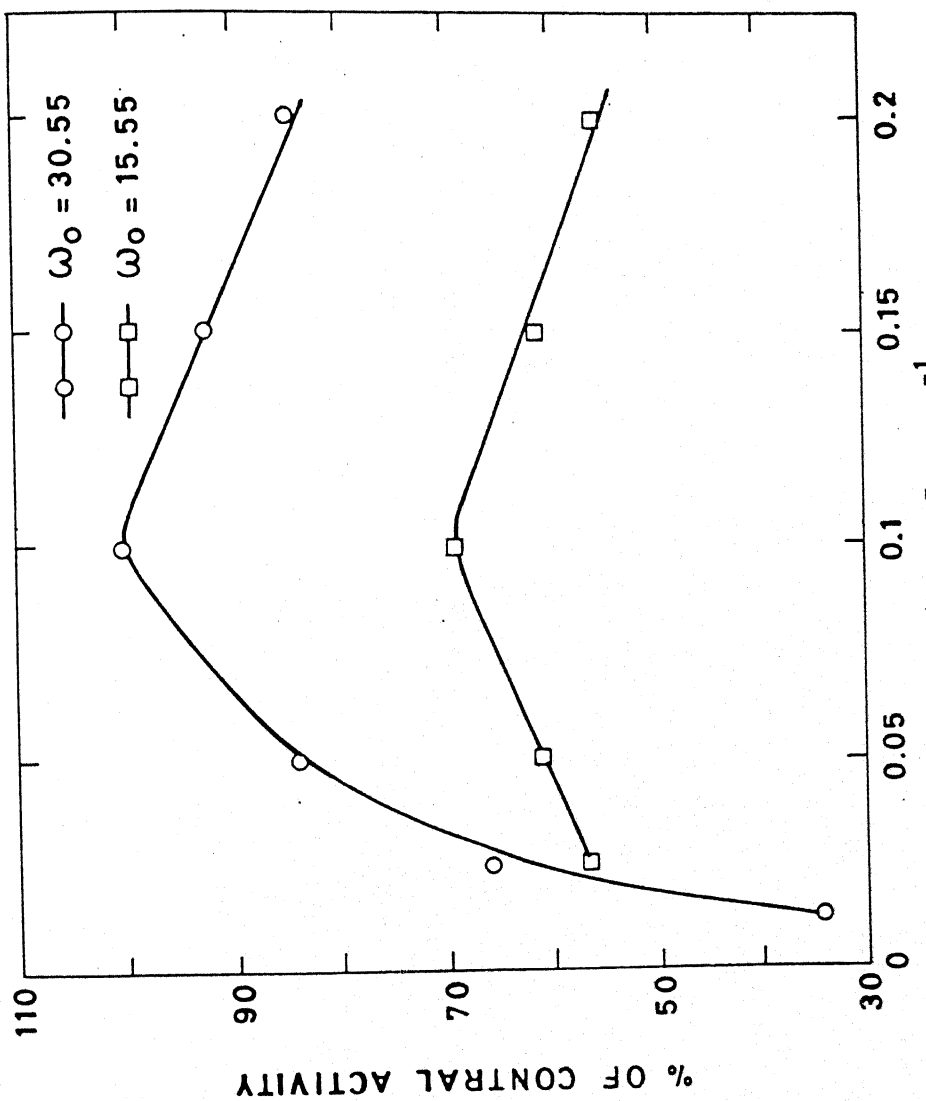


Fig.II.17. Effect of CTAB concentration on LDH activity in CTAB reverse micellar system at pH 7.0 and w_o 30.55, 15.55.

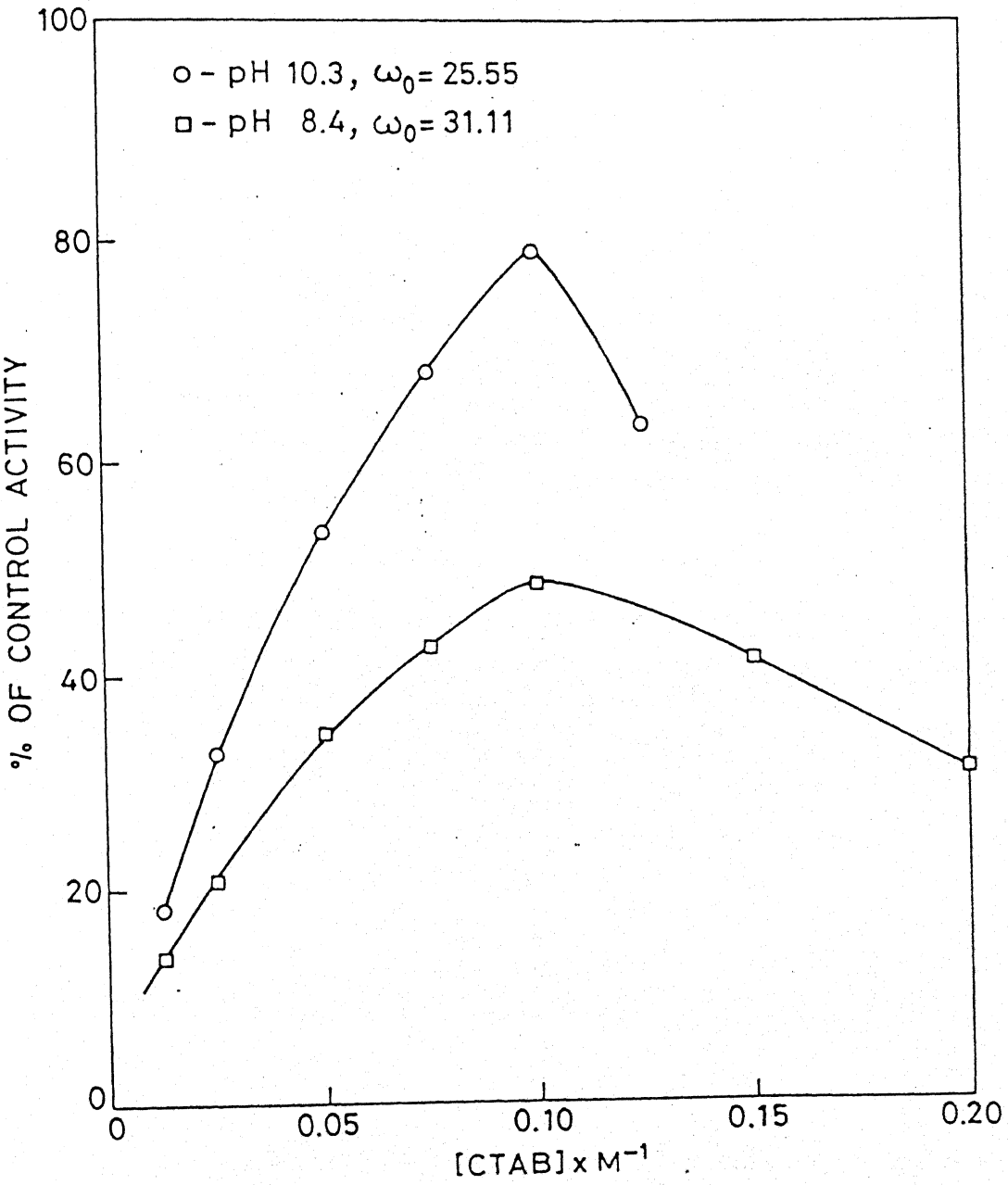


Fig.II.18. Dependence of the MDH activity on CTAB concentration at pH 8.4, w_o 31.11 and pH 10.3, w_o 25.55 in CTAB/chloroform:isooctane (1:1, v/v) reverse micellar system.

microenvironment generated by the formation of right kind of surfactant aggregates where the enzyme is able to retain its most active conformation.

In the case of LDH also water pool size affects the enzyme activity remarkably. Very low enzyme activity at lower values of w_o (0-10) may be attributed to the small water pool size where big molecules of LDH may not be able to acquire functional conformation due to volume crunch or the active site of enzyme might be distorted. This will result in the decrease in observed enzyme activity. With the increase in water pool size (w_o in the range of 10-30) enzyme apparently approaches the most functional conformation and an increase in activity is observed. Decrease in enzyme activity beyond w_o 30.55 may be because of decrease in interfacial tension at high water content. A decrease in interfacial tension may not be able to prevent all the enzyme molecules from coming in contact with organic solvent and surfactant molecules.

Similar to DHFR and LDH, the activity of MDH highly depends on the size of the water pool. Very low enzyme activity at lower water pool range (3.33-8.88) indicates that a large fraction of enzyme molecules were probably directly exposed to the organic solvent and were denatured. However, a small fraction of enzyme molecules which are protected somehow, might be in the centre of the inner core and could catalyse the reaction. It is also expected that at lower water content in the reverse micelles, the water droplet size may come close to the size of enzymes. In

yeast alcohol dehydrogenase,[27] glutathione reductase[24] etc. In most other enzymes, the pH maxima in reverse micelles becomes about 1-3 pH unit higher than the pH maxima in aqueous medium. In the case of DHFR and LDH, same optimum pH in both the media, probably indicates that in the micellar microenvironment, pK_a of amino acid residues remains almost unchanged.

The shift of the pH optimum on alkaline side for maximum MDH activity ($\approx$3 pH unit) in reverse micelles than that of aqueous solution may be attributed to the following reasons: (i) Probable charge in the ionogenic group of the solubilized enzyme. (ii) Conformational change in the enzyme on solubilization leading to apparent change in pK_a of certain amino acid residues at the active site of the enzyme. (iii) Change in the behavior of water in the water pool (or microreactor) as the reverse micelles provide unique microenvironment.[30] (iv) Ions of the surfactant CTAB which may form charged layer around the enzyme molecule. (v) Peculiar nature of the solvent in the reverse micelles. These reasons separately or simultaneously affect the local pH of the water pool of the reverse micelles.

The optimum-surfactant concentration (at which enzyme activity is maximum) is an important parameter for the study of enzymes in reverse micelles because at this concentration the surfactant aggregates formed are such that promote the enzyme in its most active conformation. This optimum surfactant concentration generates an unique microenvironment where the enzyme molecules are prevented from foreign harmful effects. At

lower surfactant concentration enzyme molecules are not well protected from the unfavourable action of organic solvents where as at higher surfactant concentration reverse micellar solution becomes too viscous. An increase in the microviscosity of the water pool will decrease the enzyme reaction rate because of the restriction in the movement of enzyme molecules in the water pool.

These data on the study of enzymes(DHFR, LDH and MDH) show that these dehydrogenase are able to retain their catalytic activity while solubilized in reverse micelles.[16,26,31] The activity profiles appear to be complex and are highly dependent on various factors like degree of hydration. pH of the injected buffer solution, surfactant concentration- etc. Our results demonstrate that the water pool size extensively regulates the catalytic activity of enzymes entrapped in reverse micelles. Once the water content reaches the proper catalytic amount, the reaction rate becomes maximum. Our results also show that number of optimum conditions in reverse micelles are more than that of aqueous optimum conditions. Some of the conditions may or may not be the same in both the media. These conditions are dependent on the size, molecular weight, subunits structure and complexity of enzymes as well as on the nature of buffers, surfactants and organic solvents. The studies on solubilization and activity of these enzymes (DHFR, LDH and MDH) in reverse micellar medium showed that these enzymes maintain their conformational integrity and subunit-subunit interaction inside the micellar core.

REFERENCES

1. Lehninger, A.L. (1984) Principles of Biochemistry, Worth Publishers, New York.

2. Dixon, M. and Webb, E.C. (1979) Enzymes IIIrd edition, Longman, London.

3. Methews, C.K., Scrimgeour, K.G. and Huennekens, F.M. (1963) Method in Enzymol 6, 364-368.

4. Stryer, L. (1975) Biochemistry, Freeman, San Francisco.

5. Hitchings, G.H. and Burchall, J.T. (1965) Advance in Enzymology 27, 417.

6. Everse, J. and Kaplan, N.O. (1973) Advance in Enzymology 37, 61-133.

7. Cahn, R.D., Kaplan, N.O., Levine, L. and Zwilling, E. (1962) Science 136, 962.

8. Ochoa, S. (1955), Method in Enzymology 1, 735-739.

9. Kitto, G.B. (1967) Biochim. Biophys. Acta 139, 16.

10. Thorne, C.J.R. (1962) Biochim. Biophys. Acta 59, 624.

11. Thorne, C.J.R. and Cooper, P.M. (1963) Biochim. Biophys. Acta 81, 397.

12. Devenyi, T., Rogers, S.J. and Wolfe, R.G. (1966) Nature 210, 489.

13. Kun, E. and Volfin, P. (1966) Biochem. Biophys. Res. Commun. 22, 187.

14. Srere, P.A. (1959) J. Biol. Chem. 234, 2544; (1961) 236, 50.

15. Martinek, K., Levashov, A.V., Klyachko, N.L. and Berezin, I.V. (1978) Dokl. Akad. Nauk. SSSR (Eng. Ed.) 236, 951-953.

16. Katiyar, S.S., Awasthi, A.K. and Kumar, A. (1988) Proc. Ind. Natl. Sci. Acad. (in press).

17. Barbaric, S. and Luisi, P.L. (1981) J. Am. Chem. Soc. 103, 4239-4244.

18. Barman, T.E. (1969) Enzyme Handbook Vol. 1, Springer-Verlag, New York.

19. Thorne, C.J.R. and Kaplan, N.O. (1963) J. Biol. Chem. 238, 1861.

20. Fendler, J.H. (1982) Membrane Mimetic Chemistry, Wiley, New York.

21. Eicke, H.F. and Rehak, J. (1976) Helv. Chim. Acta 59, 2883.

22. Zulauf, M. and Eicke, H.F. (1979) J. Phy. Chem. 83, 480.

23. Meier, P. and Luisi, P.L. (1980) J. Solid Phase Biochem. 5, 269-282.

24. Katiyar, S.S., Kumar, A. and Awasthi, A.K. (1988) Biochem. Biophys. Res. Commun. (in press).

25. Grandi, C., Smith, R.E. and Luisi, P.L. (1981) J. Biol. Chem. 256, 837-843.

26. Katiyar, S.S., Awasthi, A.K. and Kumar, A. (1988) Biochem. Int. (in press).

27. Katiyar, S.S. et al. Unpublished results.

28. Luisi, P.L. (1985) Angew. Chem. Int. Ed. Eng. 24, 439-450.

29. Smith, R.E. and Luisi, P.L. (1980) Helv. Chim. Acta 63, 2302-2311.

30. Wong, M., Thomas, J.K. and Nowak, T. (1977) J. Am. Chem. Soc. 99, 4730-4735.

31. Katiyar, S.S., Awasthi, A.K. and Kumar, A. (1988) FEBS Lett. (in press).

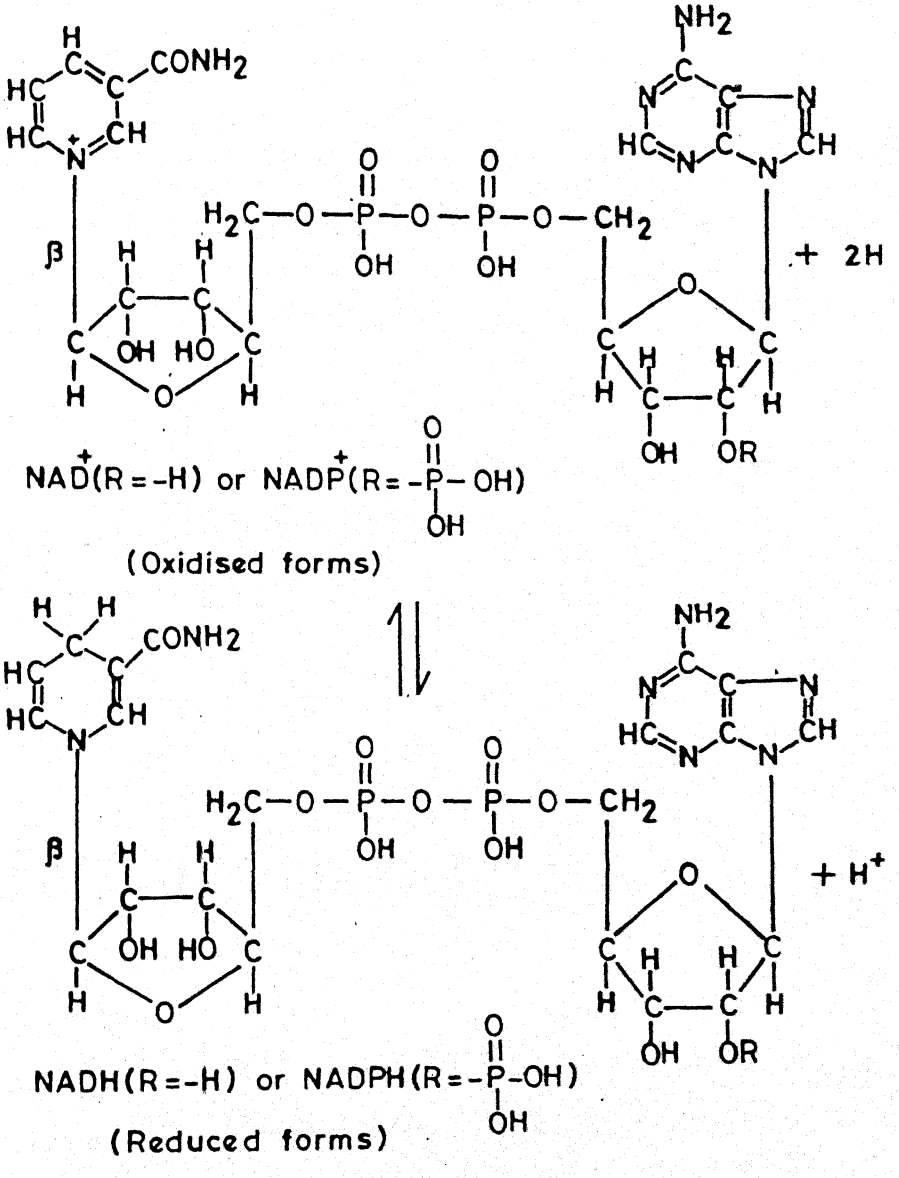
CHAPTER-III

SPECTRAL STUDY OF ENZYME REACTIONS AND STABILITY OF ENZYMES IN REVERSE MICELLES

III.1 INTRODUCTION

Spectroscopy has become a powerful tool in terms of structural information of organic and inorganic compounds. Ultraviolet/visible (u.v./vis.) infrared (I.R.), nuclear magnetic resonance (N.M.R.) and mass spectroscopy are most widely used techniques to illustrate the structural properties of given compound. Out of these U.V./vis. spectroscopy has been used most extensively for the biochemical studies.[1]

NAD and NADP (nicotinamide-adenine dinucleotide and NAD phosphate) are the two coenzymes which are involved in oxidation-reduction reactions catalyzed by dehydrogenases. Warburg and Christian showed that nicotinamide ring of these coenzymes is involved in oxidation-reduction. The overall reaction is in Scheme III.1. When the coenzymes are reduced, the ultraviolet absorption spectrum undergoes a change; the oxidised



Scheme III.1. Oxidation reduction reaction of coenzymes.

form show only a band at 260 nm due to purine and pyridine rings, but the reduced forms show an additional band at 340 nm. It has been shown that it is the nicotinamide ring which is reduced in the dehydrogenases reactions. On the basis of absorption in U.V. region, it is easily predictable that which form (either oxidised or reduced) of coenzyme is present in reaction mixture.[2]

It has been shown that spectral properties of a compound are markedly affected by the environment. Guest molecules in reverse micellar environment are therefore, expected to show significant changes in their spectral characteristics. Since enzymes, substrates and/or coenzyme-containing colloidal solutions of water in organic solvents are optically transparent, hence absorption spectroscopy is found to be a convenient tool for studying the enzyme reactions in microheterogeneous media.

The problem of time dependent stability of enzymes in reverse micellar solution requires some consideration. The optimum conditions for the stability of enzymes in reverse micelles are rather different than those in the aqueous medium. In general it appears that apolar environment destabilizes the enzyme conformation, whereas small amount of water in the reverse micelle's polar core is able to control the stability of enzyme hosted therein.[4] In fact water of the "water pool" residing in the interior of reverse micelles exhibits significantly different properties than those in bulk water.[5,6] Lower the degree of hydration (w_o) greater is the change in the properties of the water pool. This and some other factors, are responsible in the

regulation of the rigidity of enzyme conformation in reverse micelles.

In this chapter the results of spectral characteristics of reactions catalyzed by enzymes, DHFR, LDH and MDH have been presented. Their storage and operational stability in reverse micellar medium has also been studied.

III.2 EXPERIMENTAL SECTION

III.2.1 Materials

Source and purity of all the chemicals and biochemicals used in these studies have already been specified in the chapter II (See Section II.2.1).

III.2.2 Methods

Reverse micelles containing enzyme and substrates were prepared by the method described in chapter II (Section II.2.2). All spectral measurements were done on Gilford Response^{TM} u.v./visible spectrophotometer while stability studies were carried out with a Gilford model 260 u.v./visible spectrophotometer using 1 cm quartz cell at the temperature 30^{o}C $\pm$ 0.1^{o}C. After incubation of micellar solution, containing the enzyme at 30^{o}C, aliquots were taken at the indicated time and the residual activity was checked by the procedure described for the enzyme activity measurements in chapter II (see Section II.2.3).

A plot of % activity vs time gives the time dependent stability of enzyme.

III.3 RESULTS AND DISCUSSION

III.3.1 Spectral study of enzyme catalyzed reactions

It is widely accepted that the spectral properties of a compound are significantly affected by the environment in which they are studied. It is also known that guest molecules in the reverse micellar water pool, change their conformation remarkably. Due to these reasons it became necessary to study the spectral properties of reactant and products in micellar media.

III.3.1.1 Spectral study of DHFR catalyzed reaction

In the first instance, the identical nature of the enzyme reactions in water (buffer medium) and micellar solution has been established with the help of spectral data. For this, the absorption spectra of the aqueous and reverse micellar solution, before and after the completion of enzyme reactions was recorded. Fig.III.1 shows the absorption spectra of coenzymes NADPH, $NADP^+$ and the corresponding product of enzyme reaction catalyzed by enzyme DHFR. These spectra were recorded in both aqueous [Fig. III.1(a)] and reverse micellar medium [Fig. III.1(b)]. In aqueous buffers at pH 7.0 NADPH and $NADP^+$ show absorption maxima at 339.0 and 259.0 nm respectively whereas in the reverse micelles a red shift in the spectrum has been observed and it

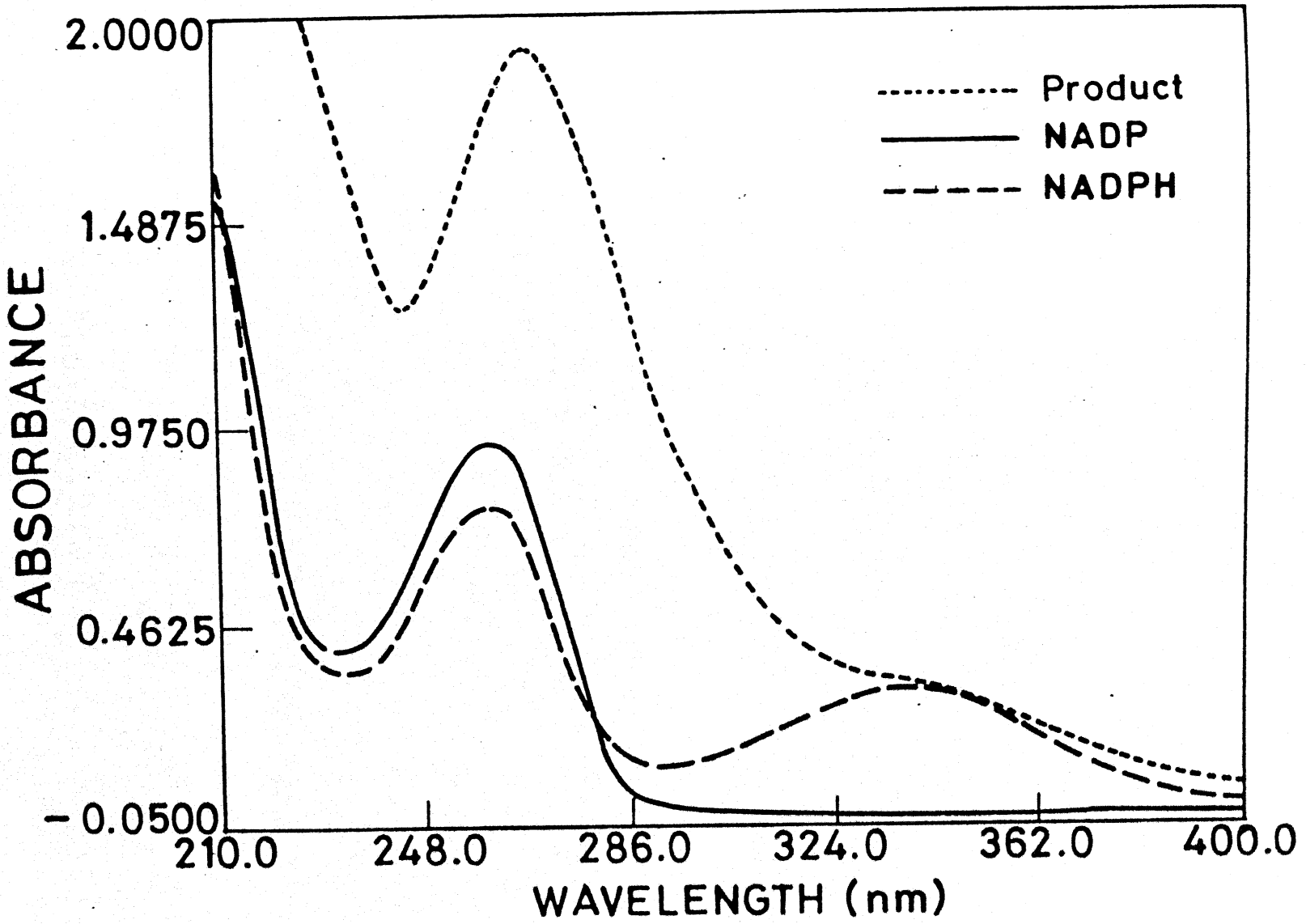


Fig.III.1(a) Electronic absorption spectra of NADPH, NADP and the product formed (after the completion of DHFR catalyzed reaction) in aqueous buffer (25 mM tris-HCl, pH 7.0).

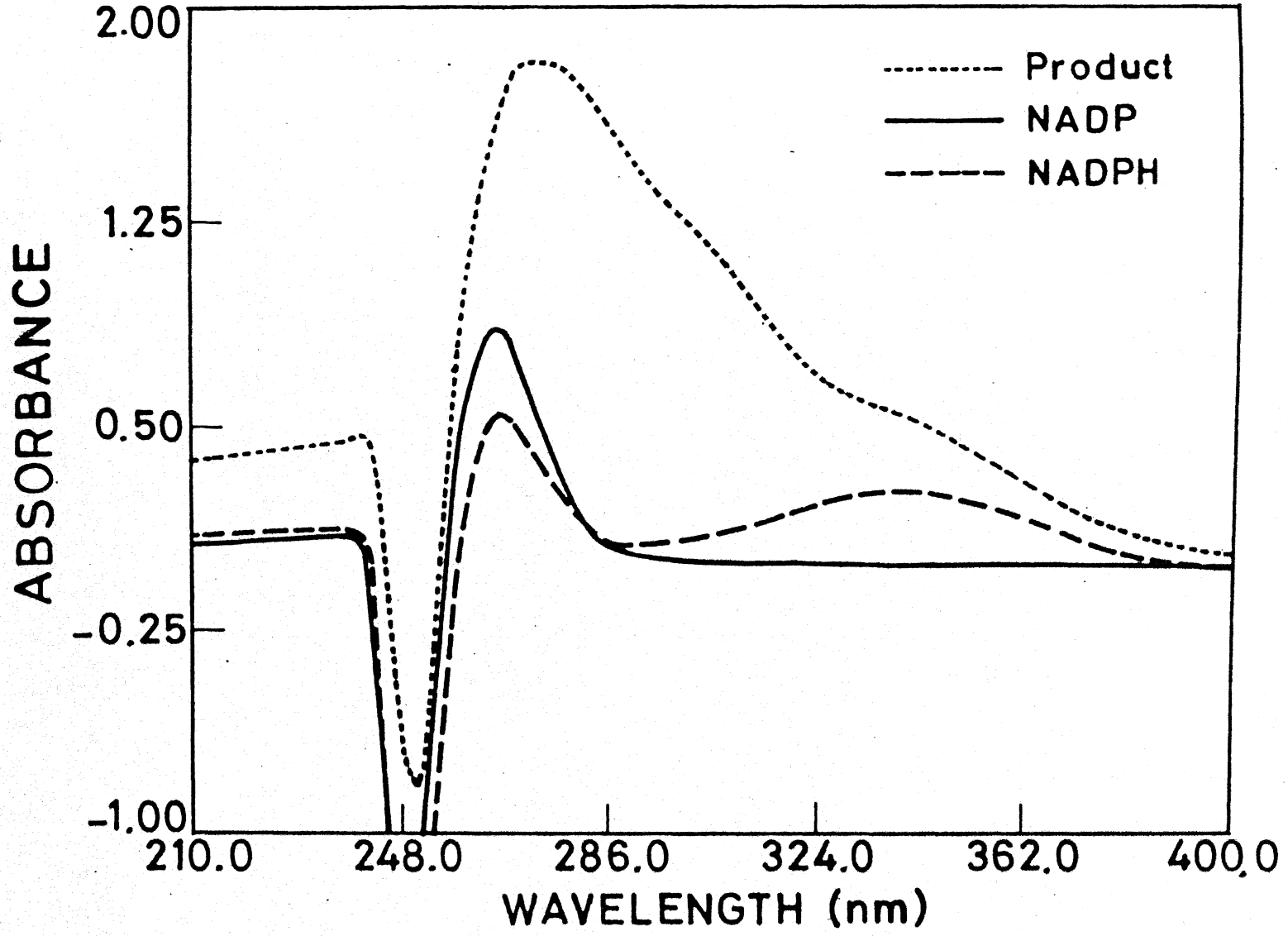


Fig.III.1(b) Electronic absorption spectra of NADPH, NADP and the product formed (after the completion of DHFR catalyzed reaction) in micellar solution of CTAB/chloroform: isooctane (1:1, v/v) at w_o 14.44; pH 7.0 (25 mM Tris-HCl).

shows absorption maxima at 341.0 and 267.0 nm. Characteristic peak of the product in aqueous solution was at 260.0 nm whereas in the reverse micelles it was 271.0 nm. It aqueous buffer peak of product was only one unit higher than the peak of the $NADP^+$ but in the case of reverse micelles it shows red shift of 5 nm. Comparison of the shape of these spectra in aqueous and reverse micellar medium shows that below 250 nm the shape of spectrum in both the media is completely different.

Fig. III.2 gives the absorption spectrum of product formed in the DHFR catalyzed reaction. These spectrum, recorded in different environment, show significant change. Spectra obtained in micellar media at two different optimum conditions for maximum enzyme activity (w_o = 14.44, pH 7.0 and w_o = 11.66, pH 9.0) are almost identical in themselves but are different from the spectrum obtained in aqueous buffer pH 7.0. Change in the reaction medium from aqueous to reverse micelles results in the red shift of absorption maxima. This red shift has also been observed when going from one optimum condition (w_o = 11.66, pH 9.0) to other condition (w_o = 14.44, pH 7.0) in the reverse micellar medium.

These data demonstrate that NADPH remains stable in the CTAB/chloroform-isooctane reverse micellar system. Red shift in the absorption maxima from aqueous medium to reverse micellar medium may be due to the change in the environment of reaction mixture and/or due to the formation of product in closed assembly. Change in the shape of spectrum from aqueous to

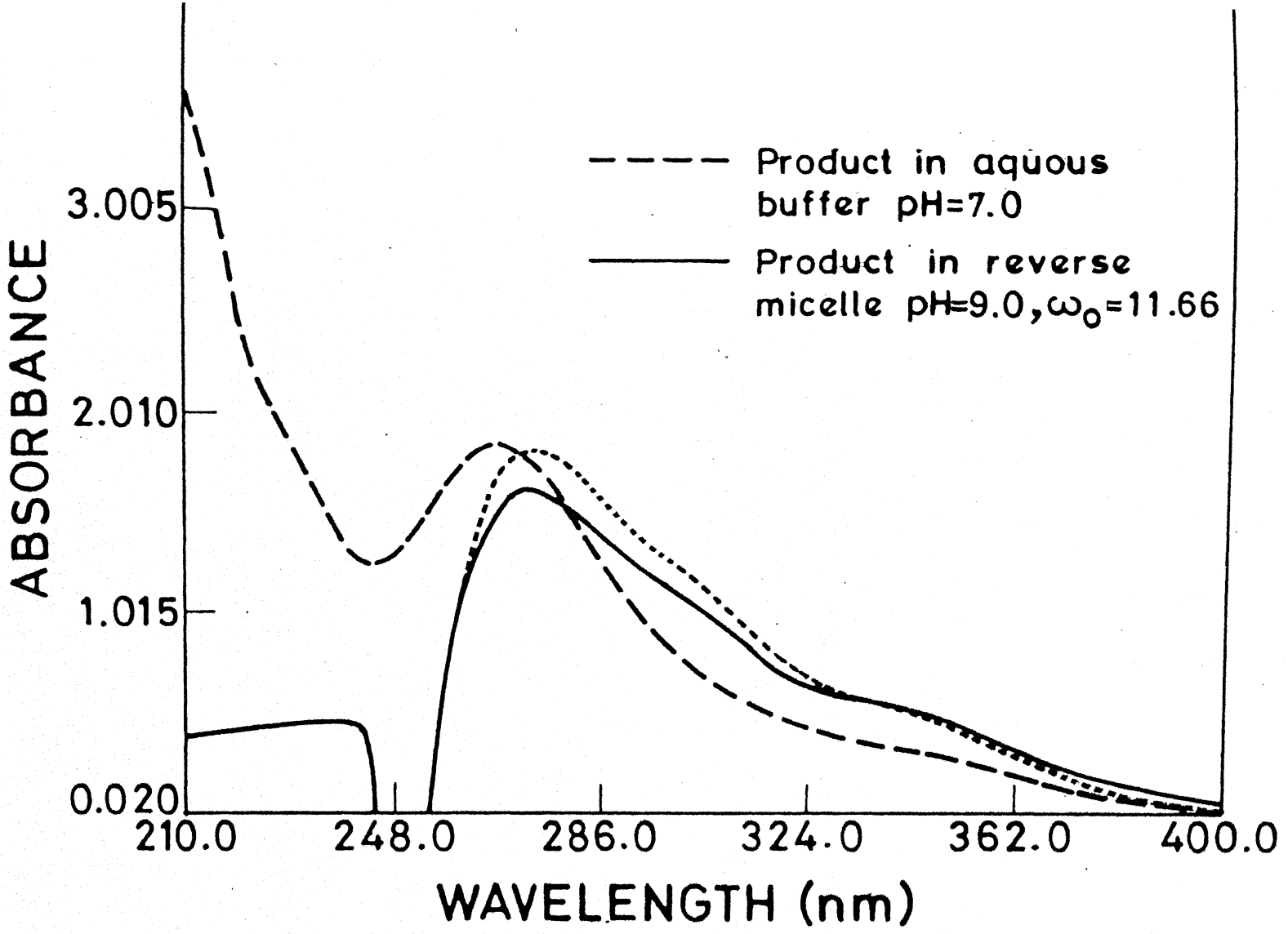


Fig.III.2. Electronic absorption spectra of the product formed(after completion of DHFR catalyzed reaction) in aqueous buffer (pH 7.0) and in micellar solution at w_o 14.44; pH 7.0 and w_o 11.66; pH 9.0. Buffers used were 25 mM Tris-HCl (pH 7.0) and 25 mM glycine-KOH (pH 9.0).

reverse micellar medium is attributed to the change from polar environment to apolar environment. These data also indicate that the decrease in absorbance at 340 nm during the assay is solely due to the enzymatic oxidation of NADPH to $NADP^+$ in the presence of dihydrofolate which simultaneously reduces to the tetrahydrofolate. These spectral data indicate that same product is formed in both the aqueous and reverse micellar media.

III.3.1.2 Spectral study of LDH catalyzed reaction

To establish the formation of same product in aqueous and reverse micellar medium for LDH catalyzed reaction absorption spectra of NADH, NAD^+, product formed, in both the media were recorded [Fig. III.3(a)&(b)]. The absorption maxima of NADH, NAD^+ and product in aqueous solution are at 339 and 259 nm, 259 nm, 260 nm respectively whereas in the reverse micellar medium these are at 339 and 264.5 nm, 265 nm, 266 nm respectively. This indicates there is a red shift of about 6 nm in these coenzymes and product in micellar media as compared to aqueous media. One peak of NADH (339 nm) remain unchanged in the micellar media. The spectrum of product in both the media is almost identical to that of authentic NAD^+.

The absorption spectra of products in the LDH catalyzed reactions are shown in Fig. III.4. The spectrum in micellar medium recorded at different values of w_o and pH (w_o = 30.55, pH 7.0 and pH 7.5; and w_o = 16.66, pH 7.5) are almost similar. All the spectra in micellar medium are almost identical to that of

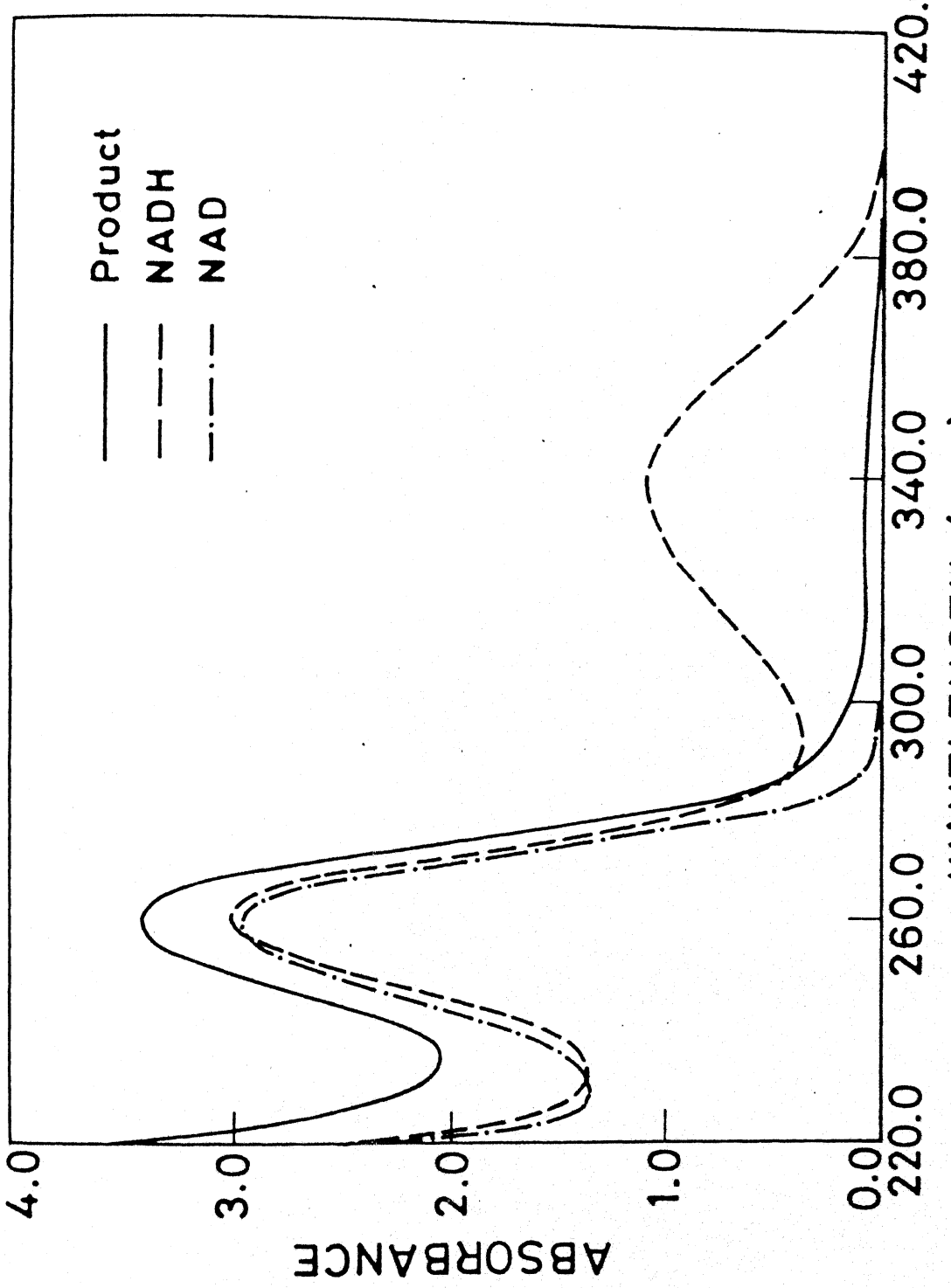


Fig.III.3(a) Electronic absorption spectra of NADH, NAD and the product formed (after completion of LDH catalyzed reaction) in aqueous buffer (100 mM potassium phosphate pH 7.0).

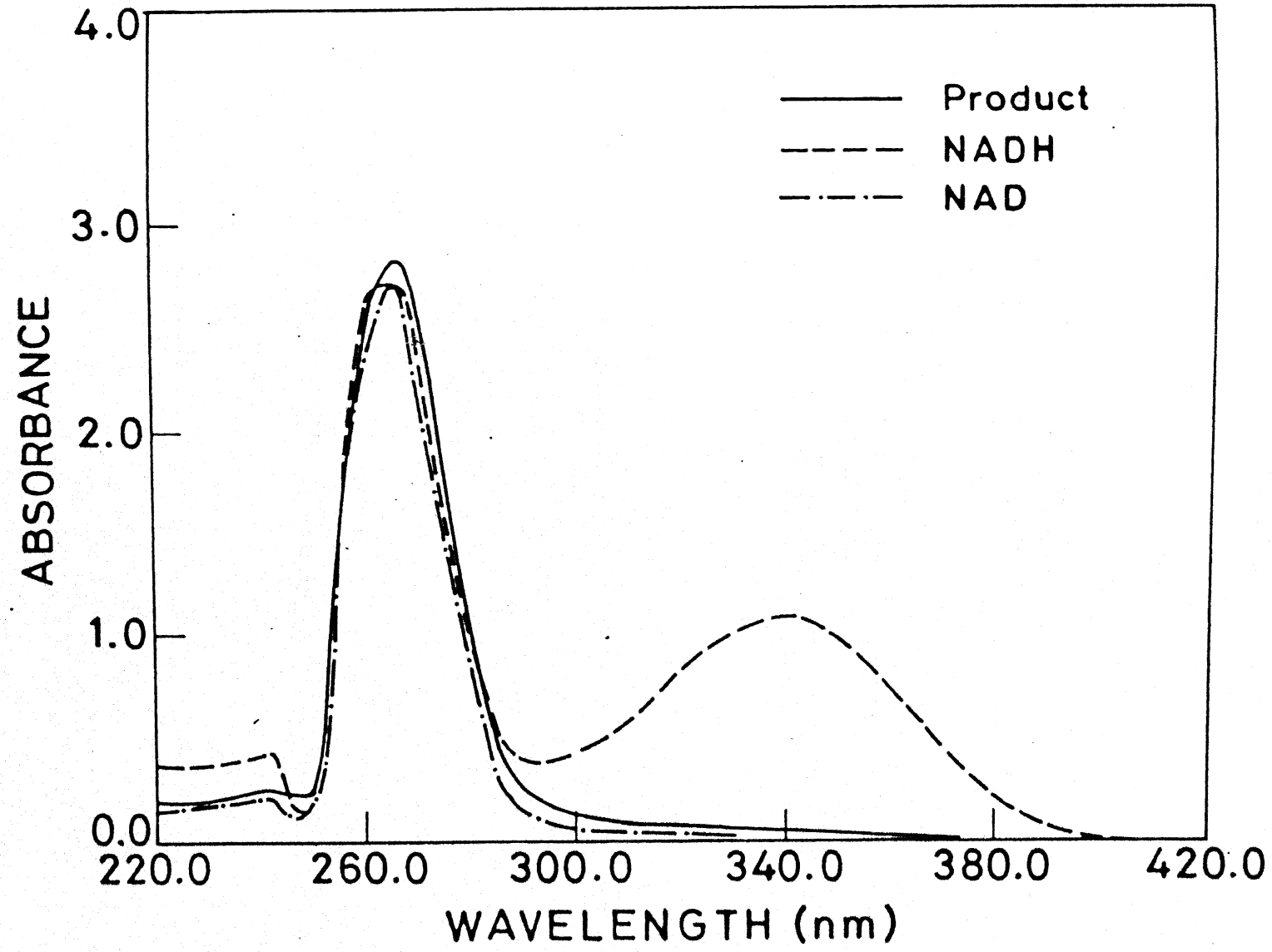


Fig.III.3(b) Electronic absorption spectra of NADH, NAD and the product formed (after completion of LDH catalyzed reaction) in reverse micellar solution of CTAB in chloroform:isooctane (1:1, v/v) at w_o 30.55, pH 7.0 (100 mM potassium phosphate).

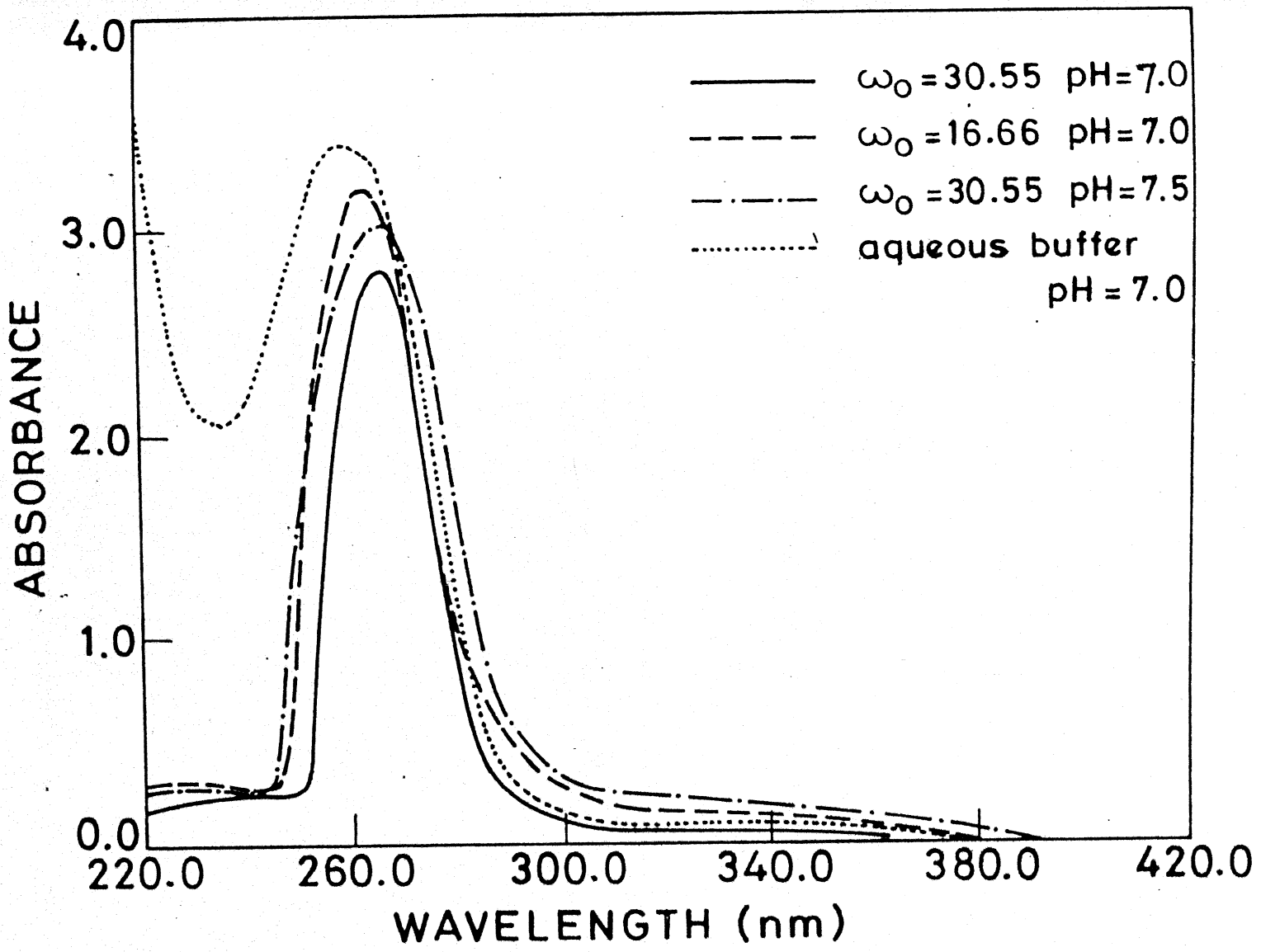


Fig.III.4. Electronic absorption spectra of the product formed (after completion of LDH catalyzed reaction) in aqueous medium (pH 7.0) and in micellar solution at w_o 30.55, pH 7.0 and 7.5; W_o 16.66, pH 7.0. Buffer used was 100 mM potassium phosphate (pH 7-8).

spectrum in aqueous solution upto about 250 nm. Below the wavelength of 250 nm, the spectra in aqueous solution and micellar medium show differences which is attributed to the change in reaction environment. These data indicate that reactant and product in micellar media remain stable. Matching of spectrum of product with NAD^+ (authentic) indicates that the enzyme LDH in reverse micelles too catalyzed the oxidation of NADH to NAD^+ in presence of sodium pyruvate.

III.3.1.3 Spectral study of MDH catalyzed reaction

Fig. III.5 illustrates the absorption data obtained for MDH catalyzed reaction. Figures III.5(a) and (b) compare the spectroscopic properties of NADH, NAD^+ and corresponding product formed during the reaction in aqueous versus micellar solution. It may be noted that near UV absorption does not change significantly in the transition from aqueous solution to a reverse micellar solution. Changes are somewhat more significant in the 250 nm region. Similar to other enzyme catalyzed reactions (catalyzed in DHFR or LDH) a remarkable red shift has been observed in change over from the aqueous solution to micellar solution. In the spectrum of product, characteristic peak of NADH (339 nm) disappears and this spectrum is almost identical to the spectrum of pure NAD^+ in both aqueous and reverse micellar medium. These spectra were recorded at identical conditions (viz. at same pH 7.5) in both the media. The waterpool size in the micellar medium corresponded to w_o = 33.33.

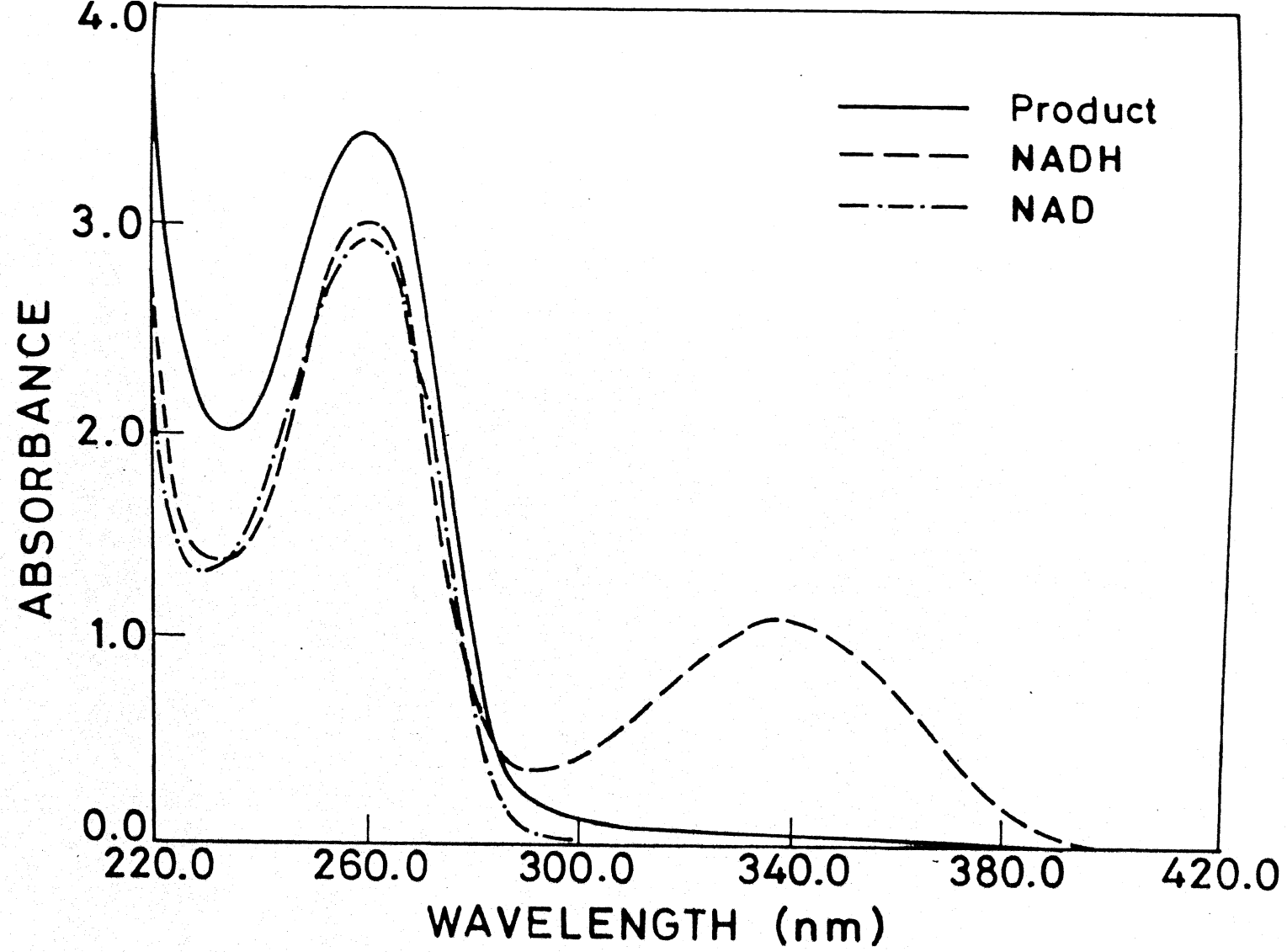


Fig.III.5(a) Electronic absorption spectra of NADH, NAD and the product formed (after completion of MDH catalyzed reaction) in aqueous buffer pH 7.5 (100 mM potassium phosphate).

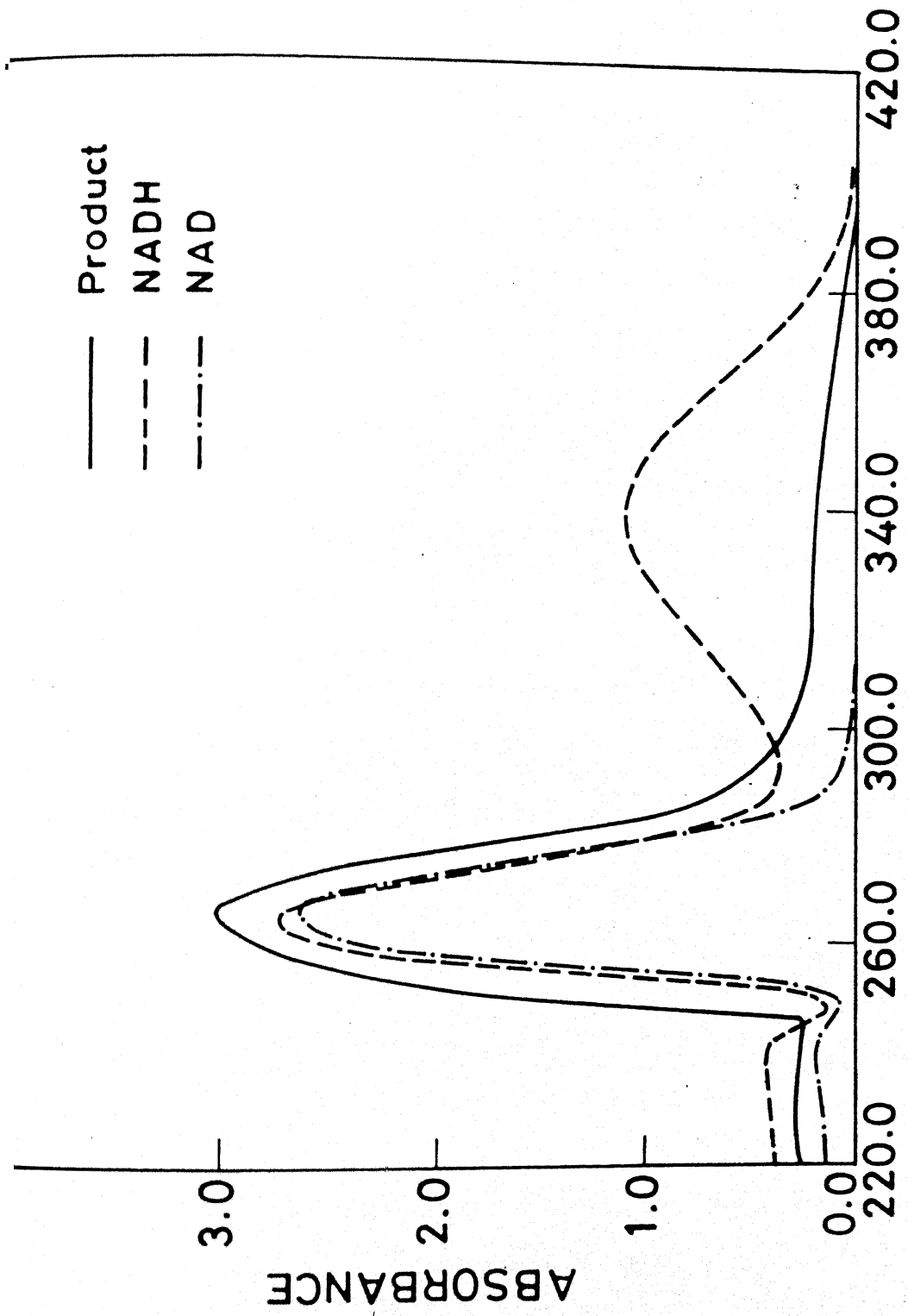


Fig.III.5(b) Electronic absorption spectra of NADH, NAD and the product formed (after completion of MDH catalyzed reaction) in micellar solution of CTAB/chloroform:isooctane (1:1, v/v) at w_o 33.33; pH 7.5 (100 mM potassium phosphate).

Spectra of the products formed in aqueous and reverse micellar medium at different conditions are shown in Fig. III.6. In the spectral region above 260 nm all the spectra are very similar, while below this region all the spectra are different from each other in as much as the U shaped region of spectrum disappears in the transition from aqueous to micellar solution. Change in pH (from pH 7.5 to pH 10.3) in aqueous medium shows slight change in the region below 260 nm while in micellar medium the variations are more significant (from w_o = 33.33, pH 7.5 to w_o = 25.55, pH 10.3).

These data demonstrate that micellar medium provides stability to enzyme reaction and that the micellar environment affects the behavior of guest molecules therein. These data also indicate that in the MDH catalyzed reaction, NADH is oxidized to NAD^+ in the presence of oxaloacetate.

Electronic absorption spectra obtained for these enzymes (DHFR, LDH and MDH) have provided valuable data to characterize and identify the reaction products in the reactions catalyzed by these enzymes. There does not appear to be perturbation in the behavior of entrapped molecule in the micellar media and it is suggested that some changes in the properties of guest molecules may be due to the performance of enzyme reaction in micro-captive environment.

III.3.2 Stability of enzymes in reverse micellar medium

Time dependent stability of enzymes in an environment is of

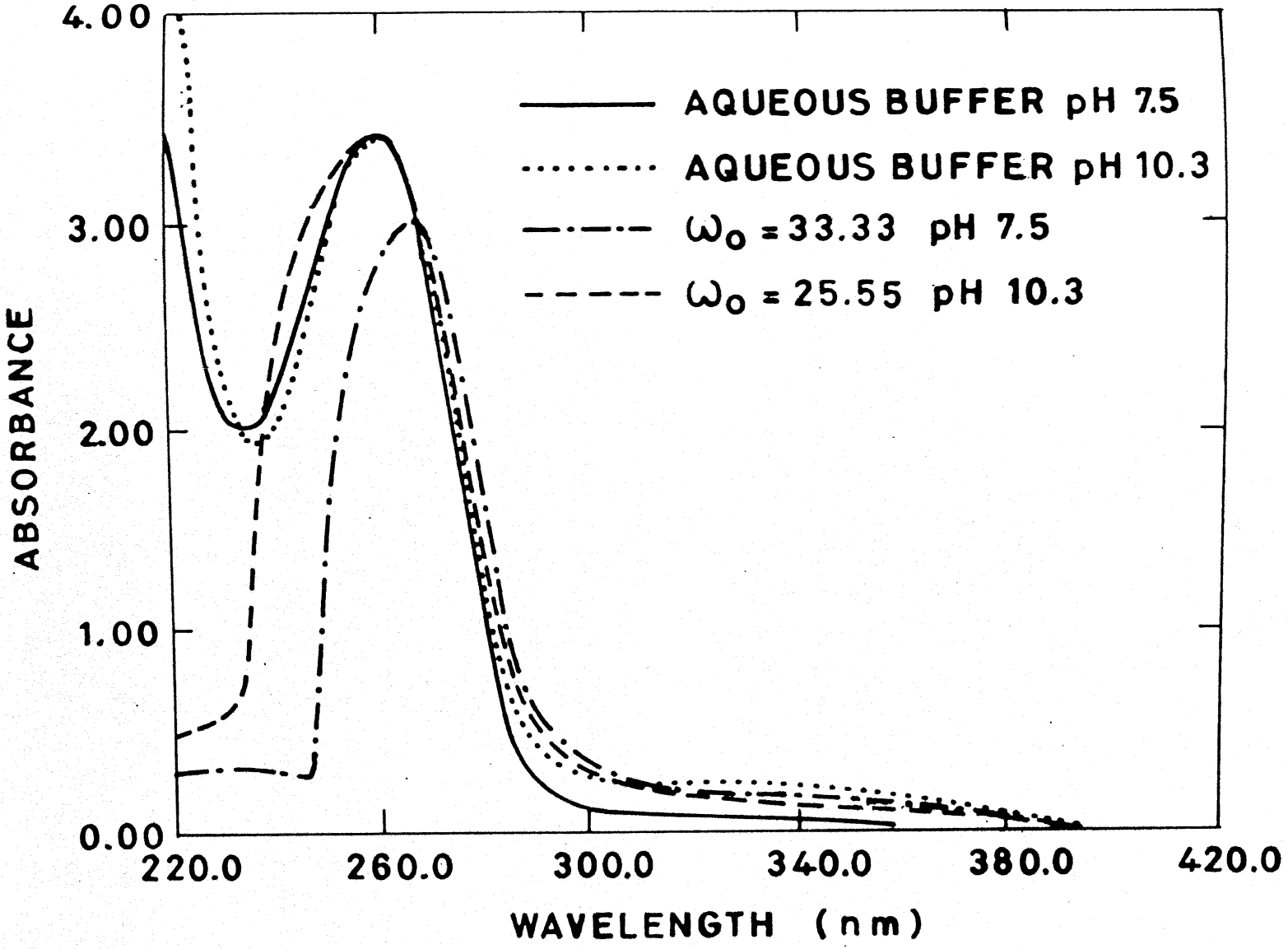


Fig.III.6. Electronic absorption spectra of the product formed (after completion of MDH catalyzed reaction) in aqueous solution (pH 7.5 and pH 10.3) and in micellar solution at w_o 33.33, pH 7.5 and w_o 25.55, pH 10.3 . Buffers used were 100 mM potassium phosphate (pH 7.5) and 100 mM glycine-KOH (pH 10.3).

great interest because it can improve the storage conditions of enzymes. It has been observed that small amount of water present in the reverse micellar core, can regulate the storage stability of enzyme and the time dependent stability of some enzymes in this medium might be much better than those stored in aqueous medium. It is possible to optimize the stability of enzymes by manipulating other parameters such as the addition of foreign substances or the decrease in temperature which is normally done to store the enzyme stock solution in aqueous medium (most of the enzymes are stored at either 0-5^{o}C or below 0^{o}C).

III.3.2.1 Time dependent stability of dihydrofolate reductase

The residual activity of DHFR as a function of time is shown in Fig. III.7. Time stability was found to be dependent on the degree of hydration (w_o) and on the presence of substrate or coenzyme in the storage medium. Fig. III.7(a) shows the change in percentage activity of DHFR as a function of time in aqueous and reverse micelles of CTAB in chloroform-isooctane mixture. The plots have been obtained at different enzyme-substrate combinations. Plots A and B give the time dependent stability in aqueous buffer (25 mM T.HCl pH 7.0). Plot A corresponds to the incubation of DHFR with NADPH whereas plot B is the result of incubation of DHFR with substrate dihydrofolate. Similarly plots C,D and E corresponds to the incubation of DHFR with NADPH, DHFR with dihydrofolate and DHFR alone respectively in the reverse micelles 100 mM CTAB in chloroform-isooctane (1:1)/water (25 mM T.HCl pH 7.0) at the w_o 14.44. From these plots it is evident

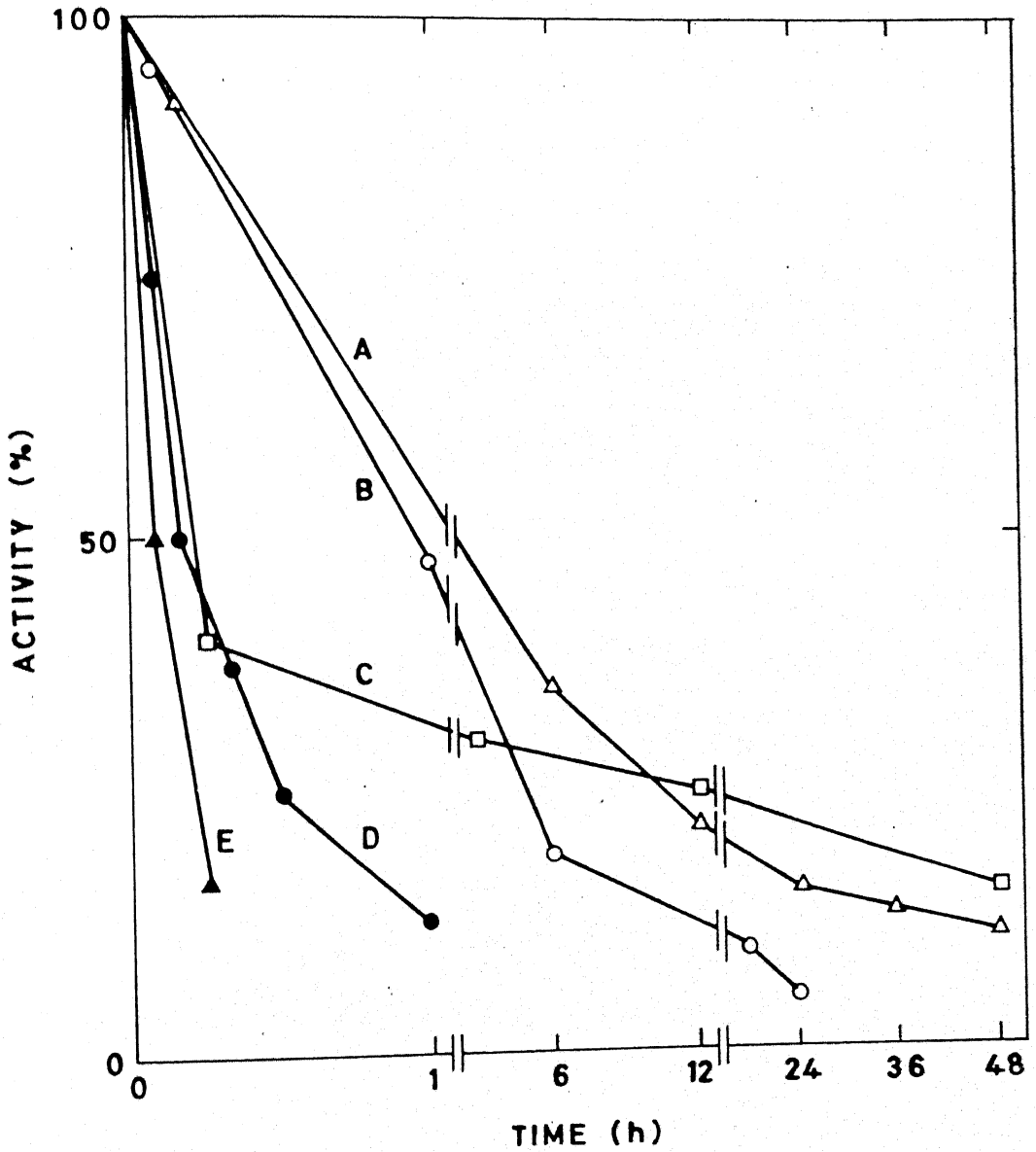


Fig.III.7(a) Stability of DHFR as a function of time in water (25 mM Tris-HCl, pH 7.0) and 100 mM-CTAB-micellar solution at w_o 14.44, pH 7.0 (25 mM Tris-HCl) in presence of substrate, coenzyme. [A] DHFR + NADPH; [B] DHFR + FAH_2 (both A and B are in aqueous medium); [C] DHFR + NADPH; [D] DHFR + FAH_2; [E] DHFR (All C, D and E are in micellar media).

that in both the media, enzyme DHFR incubated with NADPH is more stable than that of enzyme with dihydrofolate or DHFR alone. In reverse micellar media after about 15 minute incubation, enzyme alone looses about 85% of its activity whereas enzyme + dihydrofolate and enzyme + NADPH loose about 50% of their activity. On the time scale enzyme alone lost about 85% its activity within 15 minutes where as enzyme + dihydrofolate and enzyme + NADH lost the same amount of activity after about 1 hr. and 48 hrs. respectively. The possible reason for the greater stability of DHFR in presence of NADPH is the formation of strong enzyme-coenzyme complex than enzyme-substrate complex. These data indicate that coenzyme or substrate improve the stability of enzyme in both aqueous and reverse micellar media. It also shows that incubation of enzyme with NADPH provides more stable conditions that its incubation with dihydrofolate. Fig. III.7(b) shows the stability data of DHFR at different parameters such as w_o, pH and surfactant concentration. The inactivation of enzyme proceeded rapidly in micellar medium at w_o = 14.44 and 100 mM CTAB, w_o = 13.33 and 75 mM CTAB, w_o = 22.77 and 100 mM CTAB. But the stability improved as w_o was reduced to 7.22 (100 mM CTAB). At lower and medium w_o values enzyme is more stable than in aqueous buffer. After 48 hrs. in aqueous solution DHFR looses its activity by about 90% whereas the loss of enzyme activity was less in micellar medium; at w_o=13.33, CTAB, 75 mM and w_o= 14.44, CTAB 100 mM enzyme looses around 85% of its original activity after 48 hrs. whereas at w_o = 7.22 the enzyme looses about 50% of its original activity after same interval of

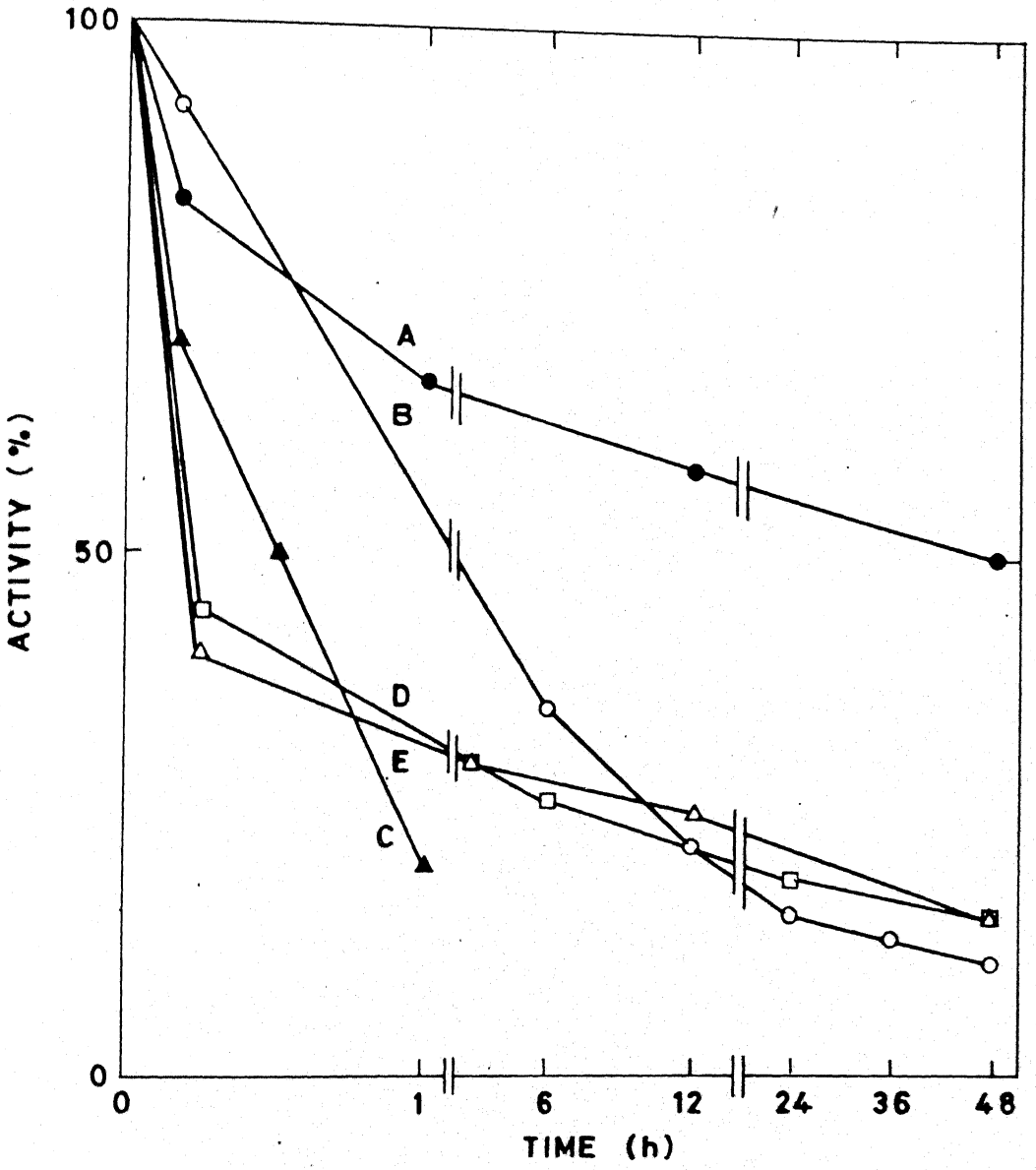


Fig.III.7(b) Stability of DHFR as a function of time in aqueous and CTAB-micellar solutions at different w_o. [A] w_o 7.22 (100 mM CTAB); [B] water, pH 7.0; [C] w_o 22.72 (100 mM CTAB); [D] w_o 13.33 (75 mM CTAB); [E] w_o 14.44 (100 mM CTAB). Buffer used was 25 mM Tris-HCl pH 7.0.

time. This type of improved stability at lower w_o values has also been observed for other enzymes like α-chymotrypsin,[4] lipase[7] etc. From these data it appears that in reverse micelles at low water content enzyme assumes a more rigid conformation and hence has a higher stability. The enzyme does not show better stability at the condition where it shows super activity. This probably indicates that DHFR has two conformation, at one conformation enzyme shows better stability while at other conformation it shows super activity. This type of different conformations for enzyme stability and activity have also been observed in aqueous medium.

III.3.2.2 Time dependent stability of Lactate dehydrogenase

Fig. III.8 gives the storage and operational stability data of enzyme lactate dehydrogenase. Effect of incubated combinations (LDH + NADH, LDH + sodium pyruvate and LDH alone) on the stability as a function of time is shown in Fig. III.8(a). Plots A and B show the stability of enzyme in aqueous buffer pH 7.0. A corresponds to the incubation of LDH with NADH whereas B for incubation of LDH with sodium pyruvate. Plots C,D and E give the variation of residual activity with time in micellar media, C for incubation of LDH with NADH at w_o = 30.55, pH 7.0 and CTAB concentration 100 mM where D and E shows the incubation of LDH with sodium pyruvate and LDH alone. The activity of LDH in 100 mM potassium phosphate buffer, pH 7.0, dropped slowly to approximately 60% often 6 hr. incubation. The

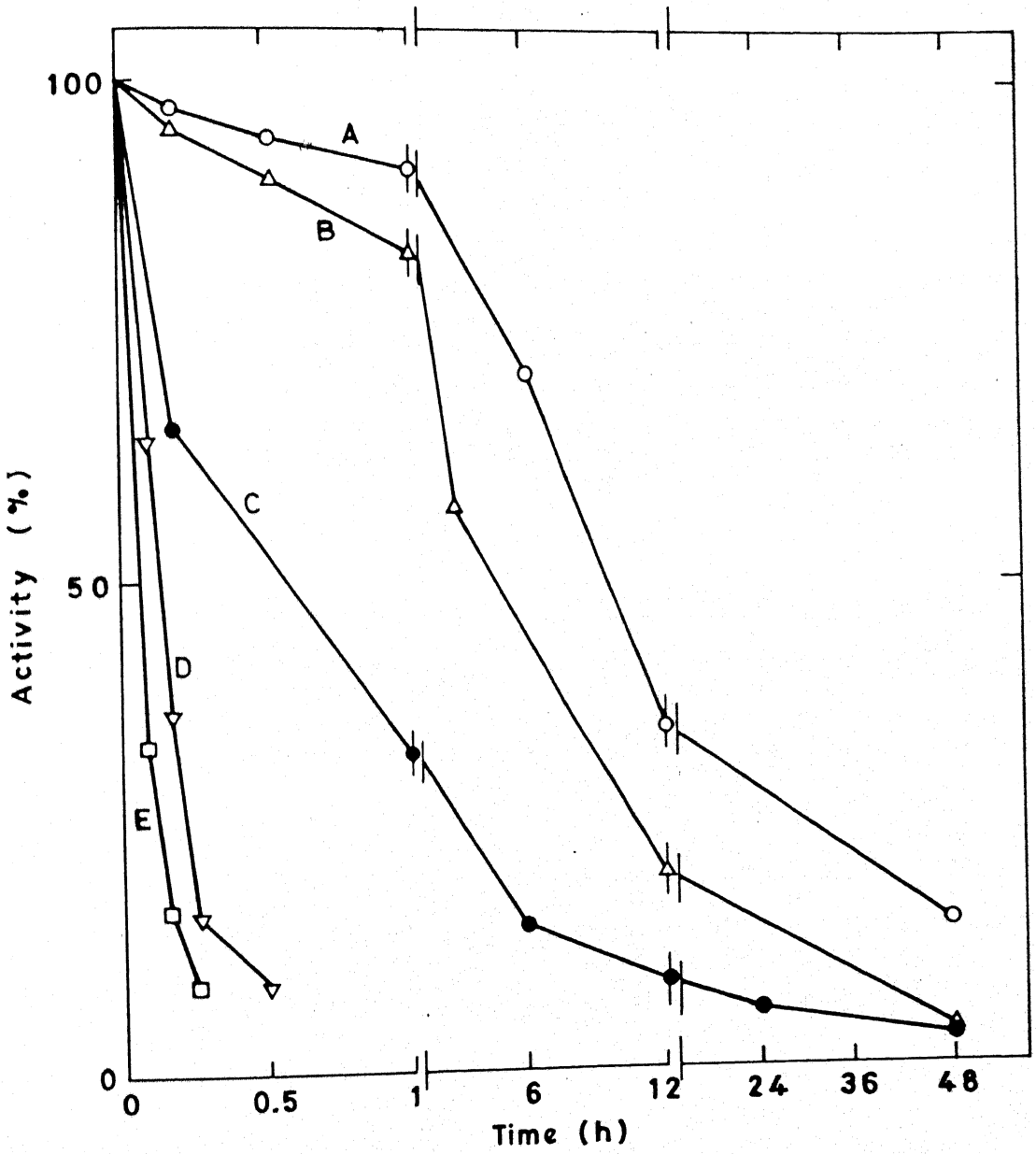


Fig.III.8(a) Stability of LDH as a function of time in aqueous and 100 mM CTAB-micellar solution in the presence of substrate and coenzyme. [A] LDH + NADH; [B] LDH + Na pyruvate (both A and B in aqueous medium pH 7.0); [C] LDH + NADH; [D] LDH + Na pyruvate; [E] LDH. (All C, D and E are in micellar media at w_o 30.55, pH 7.0). Buffer used was 100 mM potassium phosphate.

inactivation of LDH in micellar solution containing enzyme and enzyme + Na Pyruvate occurs rapidly whereas enzyme incubation with NADH shows better stability. These results demonstrate that coenzyme NADH binds to the enzyme more strongly than the binding of substrate sodium pyruvate and hence NADH provides a more rigid conformation to the enzyme molecules.

Fig. III.8(b) shows the effect of degree of hydration on time dependent stability of LDH. In micellar medium maximum stability of enzyme is at w_o = 30.55, pH 7.0 where the enzyme shows its maximum activity also. It indicates that LDH has same conformations for maximum enzyme activity and stability in micellar media. The activity of LDH in 100 mM potassium phosphate buffer, pH 7.0 dropped to approximately 15% after 48 hr. incubation whereas it was about 3% in micellar medium at w_o = 30.55, pH 7.0 after same interval of time. The enzyme is more stable in aqueous medium than in reverse micellar medium.

III.3.2.3 Time dependent stability of malate dehydrogenase

The data obtained for time dependent stability of MDH are shown in Fig. III.9. Similar to other enzymes (DHFR and LDH) this enzyme is stable in presence of coenzyme NADH in both aqueous and reverse micellar media [Fig. III.9(a)]. In micellar medium (plots C,D and E) the enzyme looses its activity very rapidly as compared to the activity in aqueous medium (plots A and B). In aqueous solution the activity of MDH dropped to about 90% after 15 minutes incubation whereas in micellar medium the

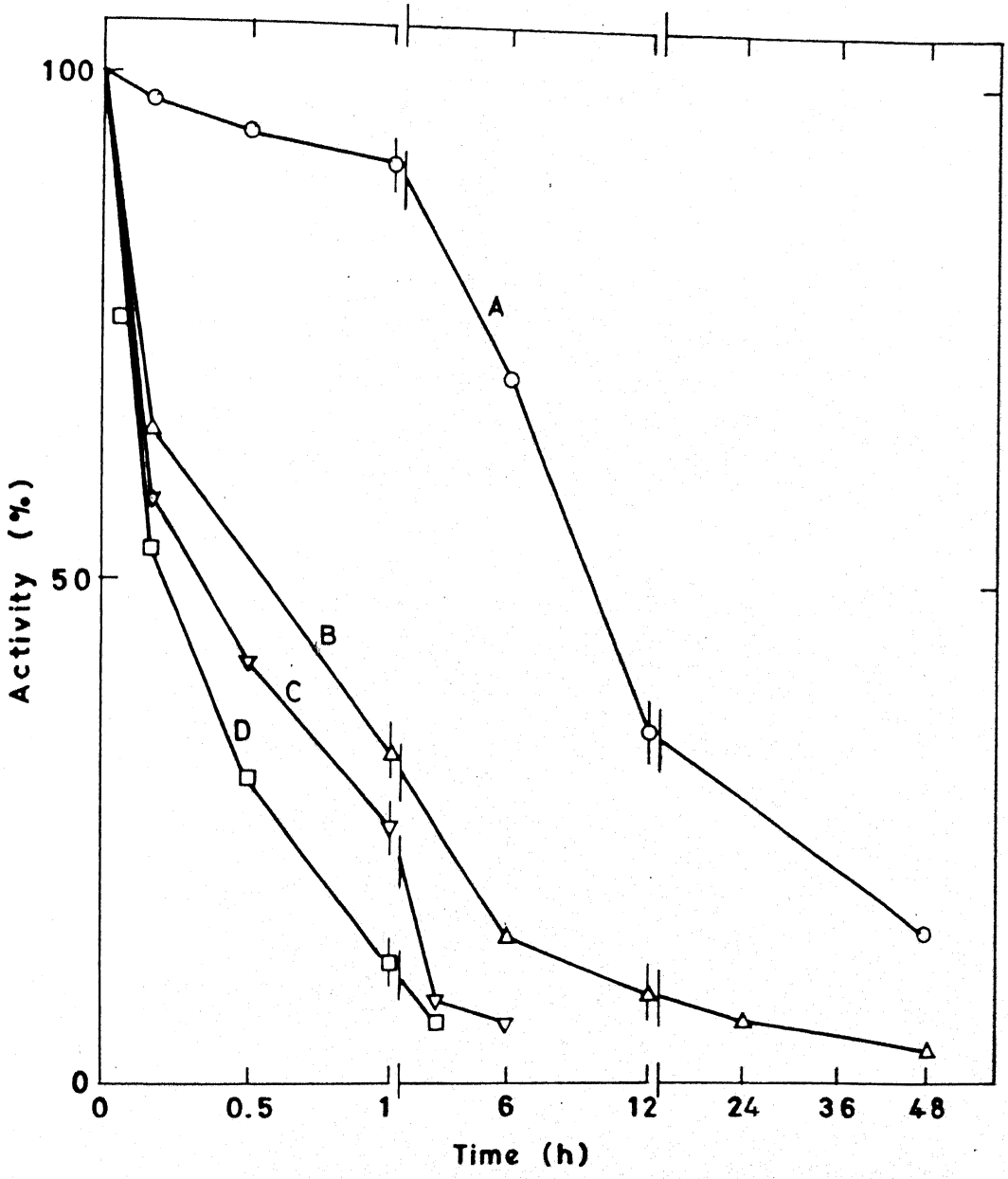


Fig.III.8(b) Stability of LDH as a function of time in aqueous and 100 mM CTAB micellar solutions at different w_o. [A] water; [B] w_o 30.55; [C] w_o 36.11; [D] w_o 16.66. Buffer used was 100 mM potassium phosphate pH 7.0.

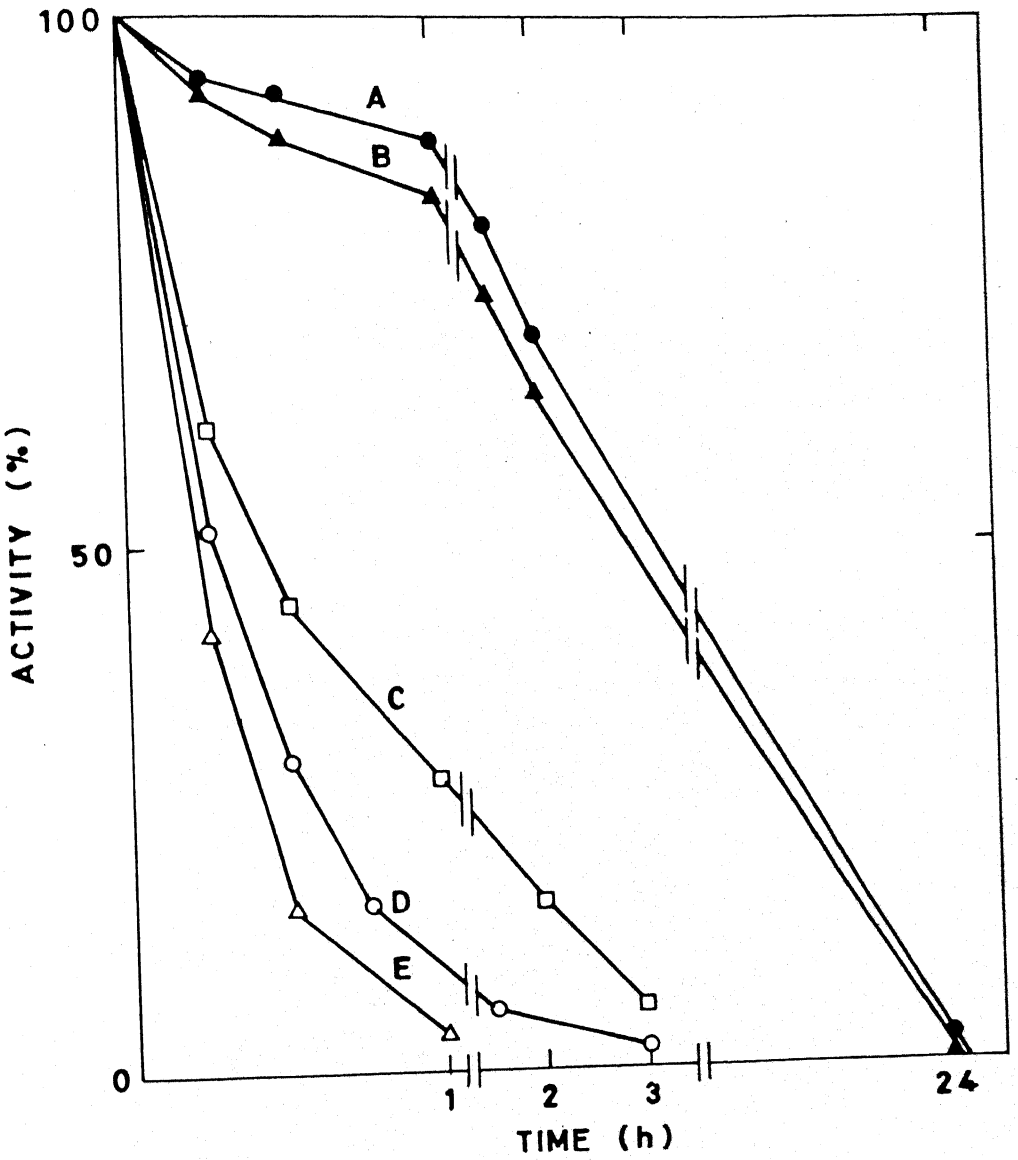


Fig.III.9(a) Stability of MDH as a function of time in aqueous and 100 mM CTAB-micellar solutions in presence of substrate and coenzyme. [A] MDH + NADH; [B] MDH + oxaloacetate (both A and B are in aqueous solution pH 7.5); [C] MDH + NADH; [D] MDH + oxaloacetate; [E] MDH. (All C, D and E are in micellar media at w_o 25.55, pH 10.3). Buffer used were 100 mM potassium phosphate (pH 7.5) and 100 mM glycine-KOH (pH 10.3).

activity dropped to about 50% after same time incubation. In micellar medium within 3 hrs. of incubation the enzyme activity reduces to almost zero value whereas in aqueous medium activity dropped to zero values after about 24 hrs. of incubation.

Fig. III.9(b) gives the effect of w_o on time dependent stability of enzyme MDH. Plots B,C and D show that in micellar medium activity is not significantly improved by changing w_o. Stability is maximum at w_o = 25.55 and pH 10.3 where the maximum enzyme activity has been observed (optimum condition). These data demonstrate that MDH in CTAB/chloroform-isooctane/water reverse micellar system is not as much stable as it is in aqueous medium. This may be due to denaturation of some fraction of enzyme molecules by apolar solvent. As it is expected some molecules of enzyme may be in contact with apolar solvents because enzyme does not show its full activity in micellar system. It shows around 80% of the activity in aqueous buffer.

The results on enzyme stability is micellar media give the information about binding of substrate and coenzyme to the enzyme. They also show that very small amount of water in the micellar core, may control the stability of enzyme in micellar media and hence give the information about the rigidity of enzyme conformation in micellar media.

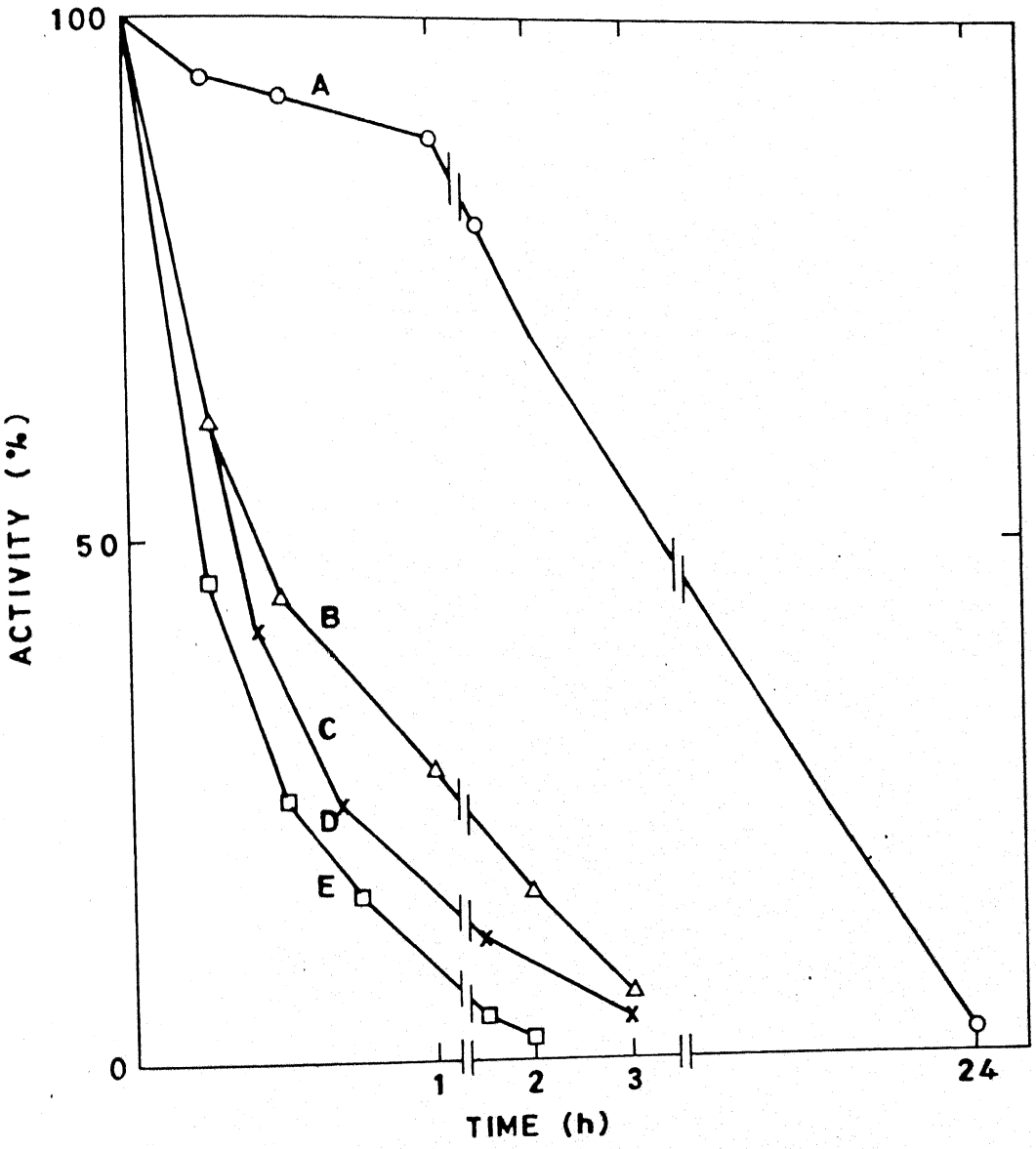


Fig.III.9(b) Stability of MDH as a function of time in aqueous and 100 mM CTAB-micellar solutions at different w_o. [A] water; [B] w_o 25.55 (pH 10.3); [C] w_o 26.94 (pH 9.5); [D] w_o 33.33; pH 7.5. Buffers used were 100 mM potassium phosphate (pH 7.5) and 100 mM glycine-KOH (pH 9.5 and 10.3).

REFERENCES

1. Williams, D.H. and Fleming, I. (1980) Spectroscopic methods in Org. Chem. IIIrd edn., McGraw-Hill, England.

2. Dixon, M. and Webb, E.C. (1979) Enzymes, IIIrd Edn., Longman, London

3. Vos, K., Laane, C. and Visser, A.J.W.G. (1987) Photochemistry and photobiology 45, 863-878.

4. Barbaric, S. and Luisi, P.L. (1981) J. Am. Chem. Soc. 103, 4239-4244.

5. Eicke, H.F. and Kavita, P. (1984) in Reverse Micelles (Luisi, P.L. and Straub, B.E. Eds.) pp. 21-35, Plenum Press, New York.

6. Gierasch, L.M., Thompson, K.F., Lacy,, J.E. and Rockwell, A.L. (1984) in Reverse Micelles (Luisi, P.L. and Straub, B.E. Eds.), pp. 265-277, Plenum Press, New York.

7. Han, D. and Rhee, J.S. (1986) Biotechnol. Bioeng. 28, 1250-1255.

CHAPTER - IV

KINETIC CHARACTERISTICS OF ENZYMES IN REVERSE MICELLES IN NON-AQUEOUS SOLVENTS

IV.1 INTRODUCTION

The characteristic property and function of enzymes is the catalysis of biochemical reactions. Catalytic function of enzymes is based on the quantitative measurement of rate of enzyme catalysed reaction. To understand the mechanism of enzyme action, kinetic studies should be correlated with chemical and structural studies on the enzymes. In order to get a complete picture of these processes, a detailed study of enzyme kinetics is necessary. Without knowledge of the kinetics of an enzyme catalyzed reaction, it is not possible to fully comprehend as to how an enzyme works in chemical terms or how it functions in the living cell.[1,2]

Many factors influence the velocity of a enzyme reaction such as enzyme concentration, concentrations of its substrate(s), activators and inhibitors specific for the enzyme, nonspecific effects of compounds (Salts and buffers), pH, ionic strength, temperature etc.[3] Effect of some of these parameters has already

been discussed in previous chapters. Out of these influencing factors, substrate concentration is one of the most important factors. In this chapter,the effect of substrate concentration on enzyme reaction rate, in aqueous medium and reverse micellar medium at all other optimum conditions, has been presented.

Enzyme activity is nearly maximum at the highest feasible substrate saturation concentration. The first step in enzyme catalysis is the binding of substrate(s) with enzyme by forming enzyme substrate complex (ES). The substrate is bound to a specific region of the enzyme called the active site. The active site is a three-dimensional entity which takes up a relatively small portion of the total volume of an enzyme. Substrates are bound to enzymes by relatively weak forces described by either lock and key model or induced fit model.[4,5]

IV.1.1 Michaelis-Menten Kinetics

At a constant concentration of enzyme, the reaction rate increases with increasing substrate concentration until a maximum velocity is reached. Michaelis and Menten interpreted the maximal velocity of an enzyme catalysed reaction in terms of the formation of a discrete ES complex. At a sufficient high substrate concentration, the catalytic sites are filled and so the reaction rate reaches a maximum. They have proposed the following scheme for the kinetic properties of many enzymes.[4,5]

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} EP \qquad .. (IV.1)$$

the catalytic rate is given by

$$V = k_{cat} \cdot [ES] \qquad .. (IV.2)$$

using steady state assumptions, the following Michaelis-Menten equation can be derived.

$$V = \frac{k_{cat}[E_o][S]}{K_m + [S]} \qquad .. (IV.3)$$

$$V = \frac{V_{max}[S]}{K_m + [S]} \qquad .. (IV.4)$$

where $V_{max} = k_{cat}[E_o]$; $K_m = \left(\frac{k_2 + k_{cat}}{k_1}\right)$ .. (IV.5)

K_m is defined as substrate concentration at which the reaction rate is half of its maximal value.

IV.1.2 Relation between dissociation constant and Michaelis constant

Dissociation constant (K_d) is given from eqn. (IV.1)

$$K_d = \frac{k_2}{k_1} \qquad .. (IV.6)$$

Michaelis constant is given by

$$K_m = \frac{k_2 + k_{cat}}{k_1}$$

$$K_m = K_d \text{ if } k_2 >> k_{cat}$$

when this condition is met, K_m is a measure of the strength of the ES complex a high K_m indicates weak binding, a low K_m indicates strong binding. The K_m indicates the affinity of the ES complex only when k_2 is much greater than k_{cat}.

At low substrate concentration i.e. [S] << K_m (eqn. IV.3) becomes as

$$V = \frac{K_{cat}[E_o][S]}{K_m} \quad .. \text{(IV.11)}$$

Above expression shows that k_{cat}/K_m is an apparent second order rate constant. The importance of k_{cat}/K_m is that it relates the reaction rate to the concentration of free, rather than total, enzyme.[4,5]

IV.1.4 Transformation of Michaelis-Menten equation in the linear form

One of the best methods for transformation of the Michaelis-Menten equation into linear form is the double reciprocal or Lineweaver-Burk plot. On inverting equation (IV.4) we get

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} \quad .. \text{(IV.12)}$$

plot of 1/V against 1/S gives a straight line with an intercept of $1/V_{max}$ and slop of K_m/V_{max}. From eqn. (IV.12) the value

of K_m and V_{max} can be obtained graphically. In the present study the kinetic parameters have been determined from Lineweaver-Burk plots.[4,5]

IV.I.5 Kinetic theory of enzyme reaction in reverse micellar systems

For a reaction between enzymes E and substrates S that obey the Michaelis kinetics in an organic solvent-water-surfactant system.[6,7]

$$E + S \rightleftharpoons ES \longrightarrow E + \text{Products} \quad .. \text{(IV.13)}$$

(i) It is assumed that a solution of a surfactant consists of two phases i.e. a bulk phase of an organic solvent and a phase of micelles wetted by water.

(ii) the substrate is distributed evently between these two phases

$$(S)_{\text{in the bulk phase}} \overset{P_s}{\rightleftharpoons} (S)_{\text{in micelles}} \quad .. \text{(IV.14)}$$

The partition coefficient, P_s of the substrate is defined in the following way

$$P_s = \frac{[S]_{micelle}}{[S]_{bulk}} \quad .. \text{(IV.15)}$$

The distribution of the enzyme need not be considered because proteins are almost insoluble in hydrophobic solvents. In addition, enzymes are as a rule denatured in non-aqueous media. Therefore it is assumed that the catalytic activity of enzyme is

concentrated only in the micellar phase.

The rate of equation (IV.13) can be expressed (in the initial instant when the concentration of products is very low as compared to the initial concentration of the substrate, with excess of substrate over the enzyme and under stationary state conditions) in the form of the Michaelis-Menten equation (IV.3).

$$V = \frac{k_{cat,micelle}[E_o]_{micelle}[S_o]_{micelle}}{K_{m,micelle} + [S_o]_{micelle}} \cdot Q \qquad .. \text{(IV.16)}$$

where Q is the volume fraction of the micellar phase

It is assumed again that the exchange of the substrate molecules between the phases is sufficiently fast i.e. enzyme reaction (IV.13) does not violate equilibrium (IV.14) and equation (IV.15) is only valid for sufficiently dilute solutions i.e. the concentration of the reagents should be much lower than that of the surfactants.

Then the concentration of the reagent can be found as

$$[S_o]_{total} = [S_o]_{micelle} \cdot Q + [S_o]_{bulk} (1-Q) \qquad .. \text{(IV.17)}$$

$$\text{and } [E_o]_{total} = [E_o]_{micelle} \cdot Q \qquad .. \text{(IV.18)}$$

On substituting equation (IV.15) in equation (IV.17)

$$[S_o]_{total} = [S_o]_{micelle} \cdot Q + \frac{[S_o]_{micelle}}{P_s} (1-Q) \qquad .. \text{(IV.19)}$$

$$\text{or } [S_o]_{micelle} = [S_o]_{total} / [Q + \frac{(1-Q)}{P_s}] \qquad .. \text{(IV.20)}$$

On substituting equation (IV.18) and (IV.20) in equation (IV.16)

$$V = \frac{k_{cat,micelle}[E_o]_{total}\cdot[S_o]_{total}}{K_{m,micelle} + [S_o]_{total}/[Q+\frac{(1-Q)}{P_s}]} \cdot 1/[Q+\frac{(1-Q)}{P_s}] \quad .. \text{(IV.21)}$$

On rearranging equation (IV.21)

$$V = \frac{k_{cat,micelle}[E_o]_{total}\cdot[S_o]_{total}}{K_{m,micelle}\cdot[Q+\frac{(1-Q)}{P_s}] + [S_o]_{total}} \quad .. \text{(IV.22)}$$

again on rearranging equation (IV.22)

$$V = \frac{k_{cat,micelle}[E_o]_{total}\cdot[S_o]_{total}}{K_{m,micelle}[1 + Q(P_s-1)]/P_s+[S_o]_{total}} \quad .. \text{(IV.23)}$$

if one assumes $k_{cat,micelles} = k_{cat,apparent}$ .. (IV.24)

and $K_{m,micelle}\ [1 + Q(P_s-1)]/P_s = K_{m,apparent}$ .. (IV.25)

then equation (IV.23) becomes

$$V = \frac{k_{cat,apparent}[E_o]_{total}\cdot[S_o]_{total}}{K_{m,apparent} + [S_o]_{total}} \quad .. \text{(IV.26)}$$

In the case of a reaction involving a charged substrate or if the substrates are confined to aqueous micellar phase i.e. $P_s >> 1$ and $P_sQ >> 1$ then

$$K_{m,apparent} = K_{m,micelle} \cdot Q \quad .. \text{(IV.27)}$$

Concerning the enzyme reactivities and their kinetic parameters in reverse micelles, Luisi et al.[8] pointed out the duality in expressing the concentration in a reverse micellar system, depending on whether one operates with water pool (wp) concentrations or with overall (ov) concentration. Corresponding to these two concentrations, there will be two K_m values to consider $(K_m)_{ov}$ and $(K_m)_{wp}$ which are numerically related by the factor f

$$(K_m)_{ov} = (K_m)_{wp} \cdot f \qquad .. \text{(IV.28)}$$

where $$f = F_w + P(1 - F_w) \qquad .. \text{(IV.29)}$$

here F_w is the water volume fraction and P_s represents the partition coefficient of the substrate (the enzyme is assumed to be soluble only in water pools)

when $P = 1$, $(K_m)_{wp} = (K_m)_{ov}$ .. (IV.30)

For substrates that are preferentially soluble in the water pool i.e. $P < 1$

Hence $(K_m)_{wp} > (K_m)_{ov}$ .. (IV.31)

and when p = o i.e. reagent that is only soluble in water pool

$$(K_m)_{ov} = (K_m)_{wp} \cdot F_w \qquad .. \text{(IV.31)}$$

Since K_m is a good measure of the dissociation constant of the enzyme-substrate (ES) complex, therefore it becomes all the more necessary to decide that which K_m (either $(K_m)_{wp}$ or $(K_m)_{ov}$] is the physically relevant one. In general for most of the enzymes,

it is found that the value of $(K_m)_{ov}$ is closer to $(K_m)_{aq}$ and is generally more than an order of magnitude smaller than $(K_m)_{wp}$.

IV.2 EXPERIMENTAL SECTION

IV.2.1 Materials

Source and purity of all the chemicals used in the present study has already been mentioned in the previous chapter.

IV.2.2 Methods

The methods for the preparation of enzyme and substrate containing reverse micelles and measurements of enzyme reaction rate were similar to those described in chapter II. Dependence of the initial velocity on the substrate concentrations have been investigated by varying the concentration of substrates while keeping other parameters, which influence enzyme reaction rate, constant. Michaelis constant (K_m), and other kinetic and binding parameters have been determined from Lineweaver-Burk (double reciprocal) plots.

IV.3 RESULTS AND DISCUSSION

IV.3.1 Michaelis-Menten Kinetics

Investigations on the dependence of the enzyme reaction rate with substrate concentration in reverse micellar medium show that these enzymes (DHFR, LDH and MDH) follow Michaelis-Menten kinetics. Fig IV.1 shows the effect of NADPH concentration on DHFR

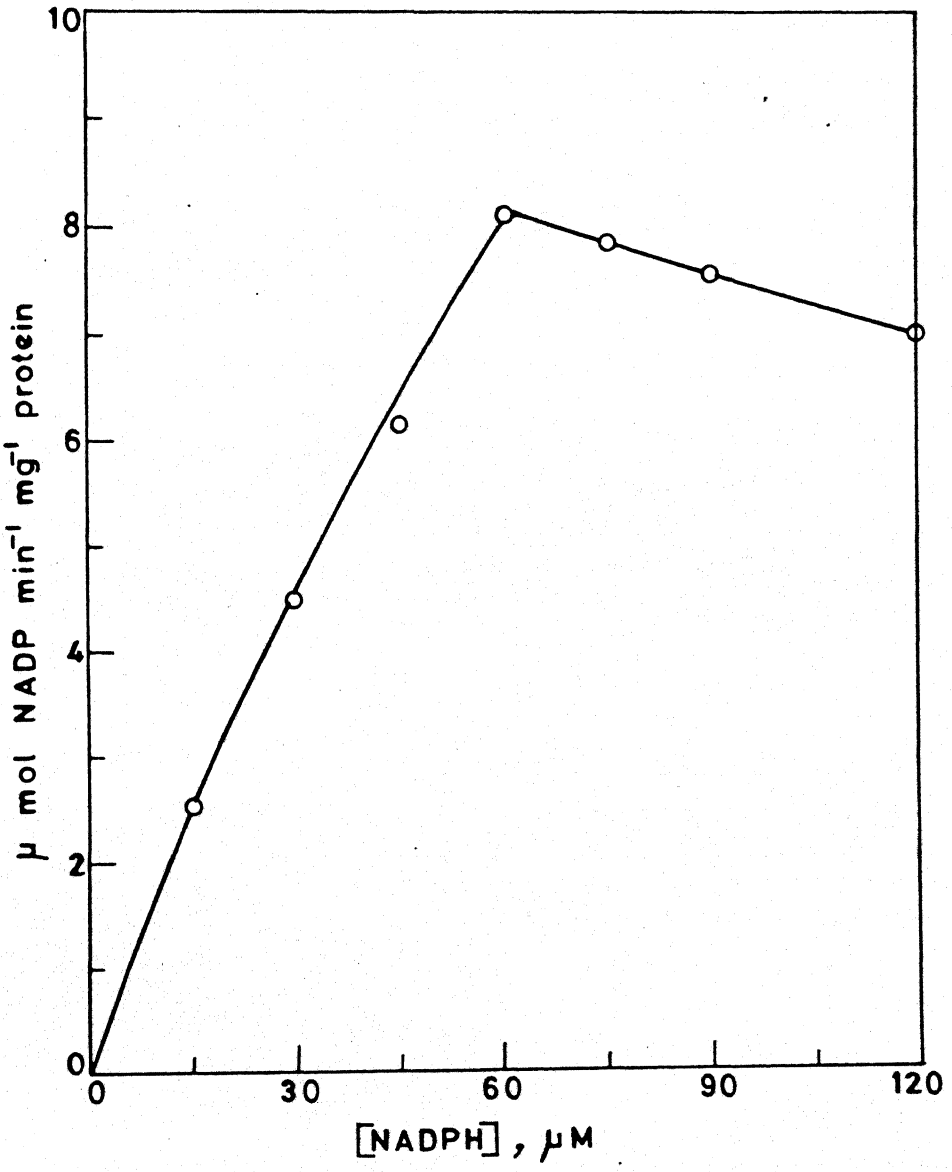


Fig.IV.1. Dependence of DHFR activity on NADPH concentrations in 100 mM CTAB/chloroform:isooctane (1:1, v/v) at w_o 14.44 and pH 7.0. FAH_2 concentration was 60 μM and buffer was 25 mM Tris-HCl.

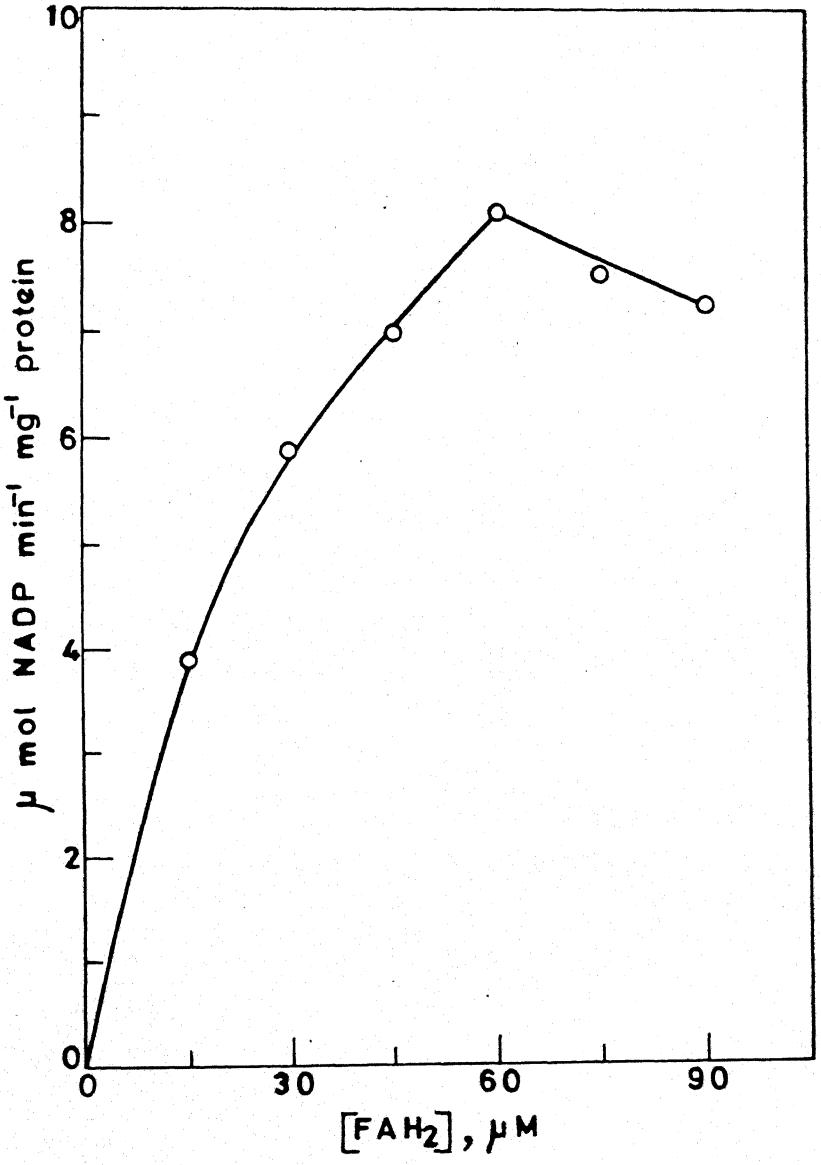


Fig.IV.2. Effect of FAH_2 concentrations on DHFR activity in CTAB/chloroform-isooctane micellar system at w_o 14.44 and pH 7.0. The concentrations were: [CTAB] = 100 mM; [NADPH] = 60 μM; [buffer, Tris-HCl] = 25 mM.

activity at optimum conditions and constant (saturation) FAH_2 concentration. Enzyme activity increases almost in linear fashion and maximum activity was found at 60 uM NADPH concentration. The effect of substrate FAH_2 concentration on the activity of DHFR at constant and saturation concentration of NADPH is shown in Fig. IV.2. The effect of FAH_2 concentration seems to be similar to NADPH concentration, showing maxima at a concentration of 60 uM.

The effect of NADH and sodium pyruvate concentration on the activity of lactate dehydrogenase at optimum conditions is shown in Fig. IV.3 and in Fig. IV.4 respectively. The enzyme activity increases with increasing concentration of substrates up to 0.2 mM of NADH and 1 mM of Na pyruvate concentration.

Fig. IV.5 shows MDH activity vs. NADH concentration profile at optimum conditions and fix concentration of oxaloacetate at saturation. The increase in NADH concentration results in linear increase in MDH activity up to 0.2 mM NADH concentration. Effect of substrate oxaloacetate concentration on MDH activity is shown in Fig. IV.6. The enzyme shows maximum activity at 0.5 mM oxaloacetate concentration. These plots showing the effect of substrate concentration on enzyme activity demonstrate that Michaelis-Menten Kinetics is followed by these enzymes in reverse micelle. At high substrate or coenzyme concentration the deviation from Michaelis-Menten kinetics occurs because of substrate inhibition which will be discussed in section IV.3.2.

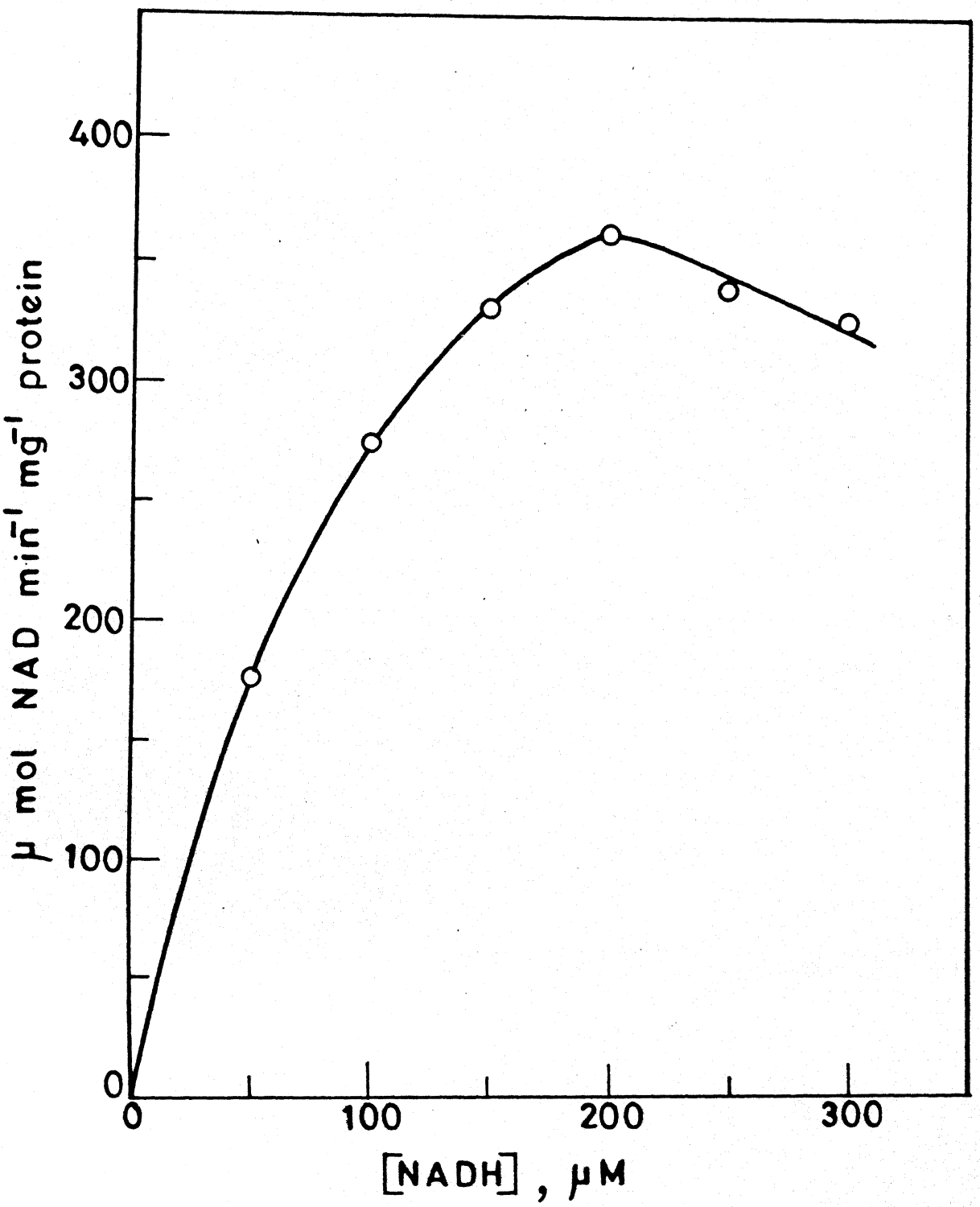


Fig.IV.3. Variation of LDH activity with NADH concentrations in CTAB micellar system at w_o 30.55, pH 7.0. The concentrations were : $[CTAB]^o$= 100 mM; [Na pyruvate]= 1 mM. The buffer was 100 mM potassium phosphate.

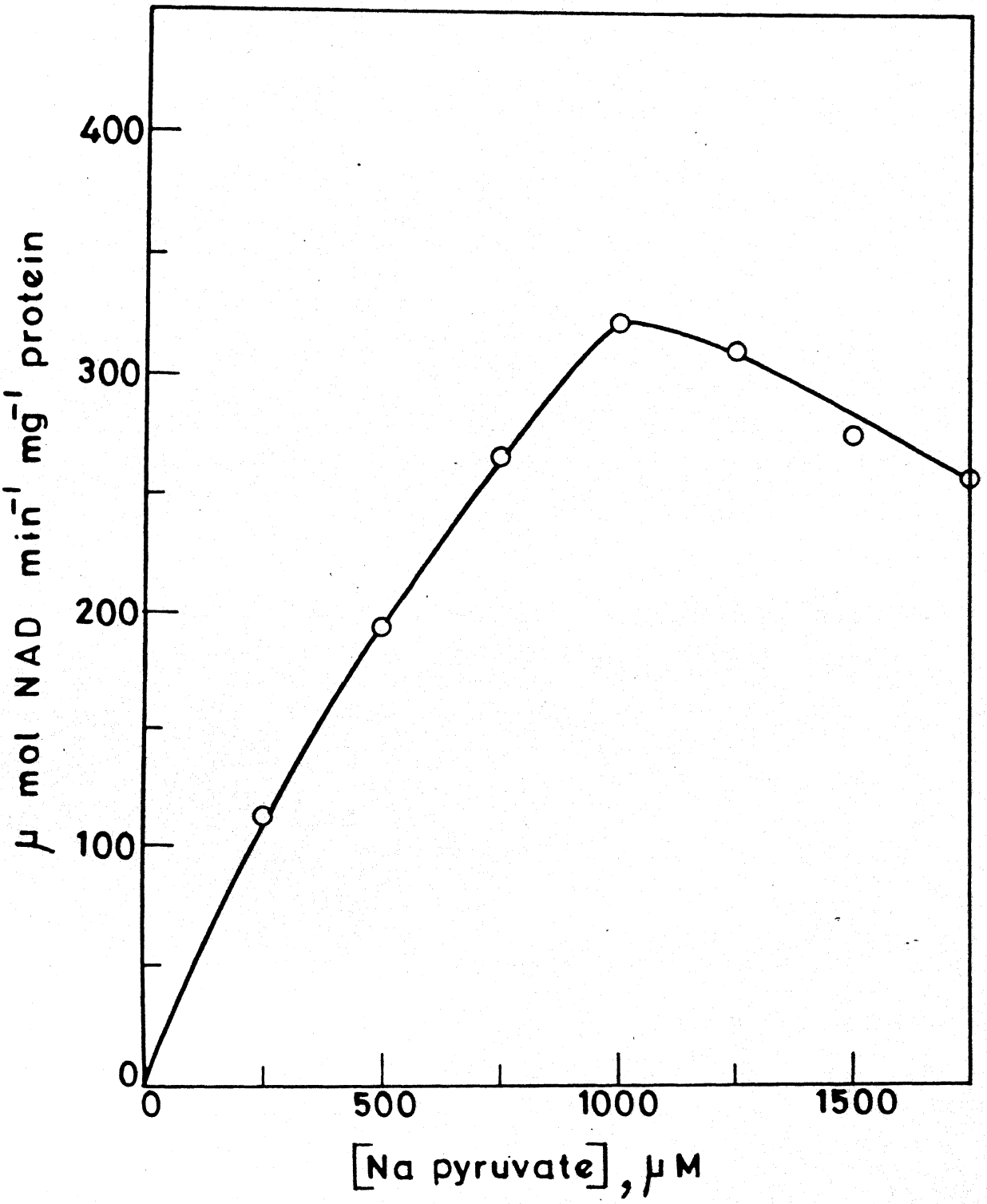
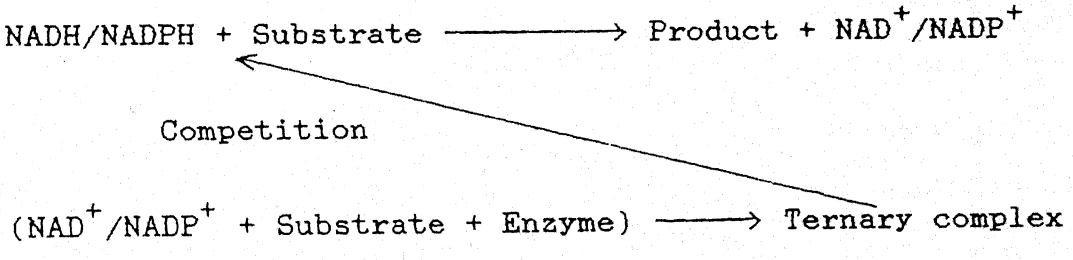


Fig.IV.4. Effect of Na pyruvate concentrations on LDH activity in reverse micellar system of CTAB in chloroform: isooctane (1:1, v/v) at w_o 30.55, pH 7.0. The concentrations were [CTAB] = 100 mM, [NADH] = 0.2 mM. The buffer was same as in Fig. IV.3.

IV.3.2 Phenomenon of substrate inhibition

To overcome the problem of product inhibition and approach to equilibrium, generally high concentration of substrate is used. But some times very high substrate concentration results in the fall of enzyme activity which is called the phenomenon of substrate inhibition. Due to this phenomenon hyperbolic kinetics with an asymptote V_{max} may not apply.[3] In the case of dihydrofolate reductase assayed in the direction of tetrahydrofolate formation, NADPH or dihydrofolate concentrations higher than about 60 uM lead to a lowering of initial rate (Figs. IV.1 and 2). Similarly, the data of Figs. IV.3 and 4 gives the occurrence of substrate inhibition for lactate dehydrogenase. LDH was assayed in the direction of lactate formation. When the coenzyme (NADH) concentrations became more than 0.2 mM or pyruvate concentrations became more than 1 mM, LDH activity decreased from its maximum value. In an identical fashion when malate dehydrogenase was assayed in the direction of malate formation, NADH concentrations higher than 0.2 mM or oxaloacetate concentrations higher than 0.5 mM resulted in the display of reduced enzyme activity. This phenomenon of substrate inhibition may be attributed to the formation of tight substrate-$NADP^+$ or NAD^+ enzyme abortive complex. Possible mechanism of substrate inhibition is given below



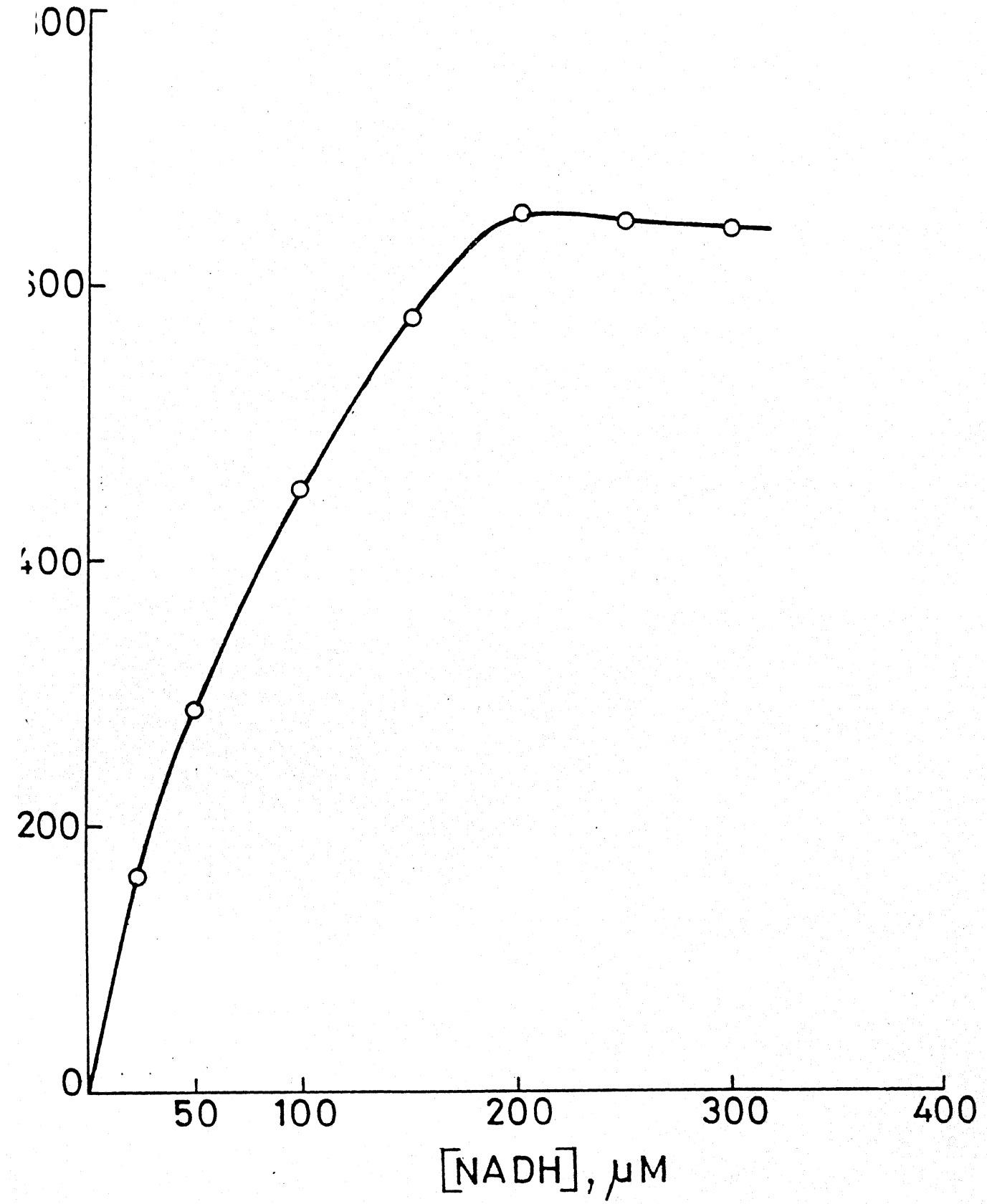


Fig.IV.5. Dependence of MDH activity on NADH concentrations in CTAB micellar system at w 25.55, pH 10.3. The concentrations were: [CTAB] = 100 mM, [Oxaloacetate] = 0.5 mM. The buffer was 100 mM Glycine-KOH.

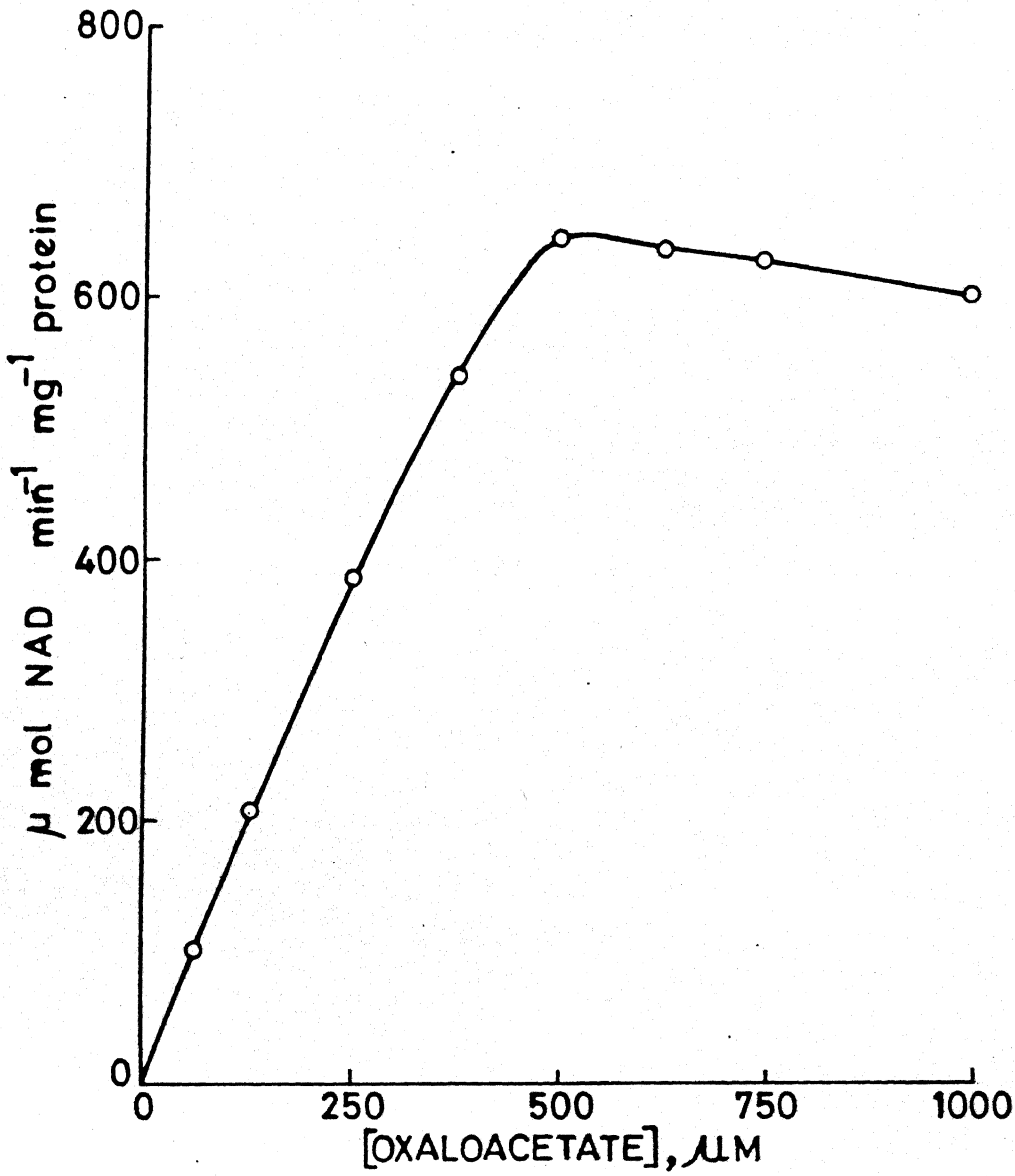


Fig.IV.6. Variation of MDH activity with oxaloacetate concentrations in 100 mM CTAB/chloroform:isooctane (1:1, v/v) at w_o 25.55, pH 10.3. The NADH concentration was 0.2 mM. Buffer was same as in Fig. IV.5.

Other factors such as high substrate concentration in water pool, enzyme reactions at very low water content in the reverse micellar microenvironment may also influence the rate.

IV.3.3 Lineweaver-Burk Plots: Determination of kinetic and binding properties of enzymes

Fig. IV.7 shows the variation of inverse of DHFR catalyzed reaction rate with inverse of NADPH concentration at four FAH_2 concentrations. These plots are linear and meet on X-axis. Double reciprocal plots of DHFR reaction rate and dihydrofolate concentration at different NADPH concentration are given in Fig. IV.8. Data for these primary plots of Fig. IV.7 and 8 were obtained by observing the effect of substrate and coenzyme concentrations on DHFR activity at w_o = 14.44, pH 7.0 and 100 mM CTAB concentration. The Michaelis constant (K_m) for dihydrofolate and NADPH were determined from the secondary plots obtained by plotting intercept on 1/V axis of one substrates against reciprocal of concentration of other substrate (Fig. IV.9). Fig. IV.10 and IV.11 show the Lineweaver-Burk plots for DHFR catalysed reactions at a different water pool viz. w_o = 7.22, pH 7.0 and 100 mM CTAB concentration. The intercept obtained on 1/V axis in Fig. IV.10 and IV.11 are plotted against inverse of substrate concentrations to obtain the secondary plots shown in Figs. IV.12(a) and (b). From these plots K_m values were determined. Table IV.1 summarizes the data on kinetic and binding properties (K_m, V_{max} and K_d) of dihydrofolate reductase

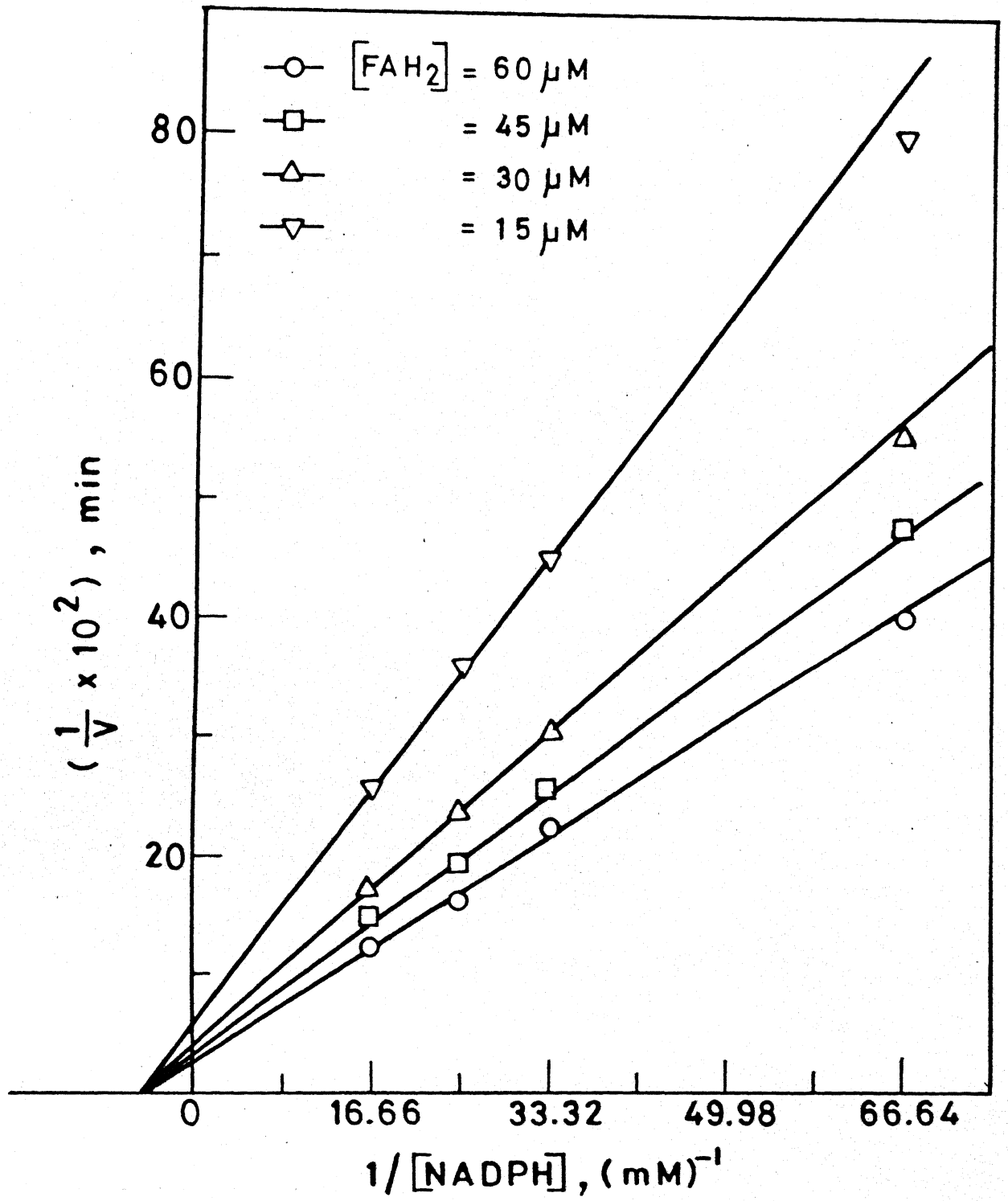


Fig.IV.7. Lineweaver-Burk plots for initial DHFR rate with NADPH concentrations in CTAB micellar system at different FAH_2 concentrations and w_o 14.44, pH 7.0, 100 mM CTAB.

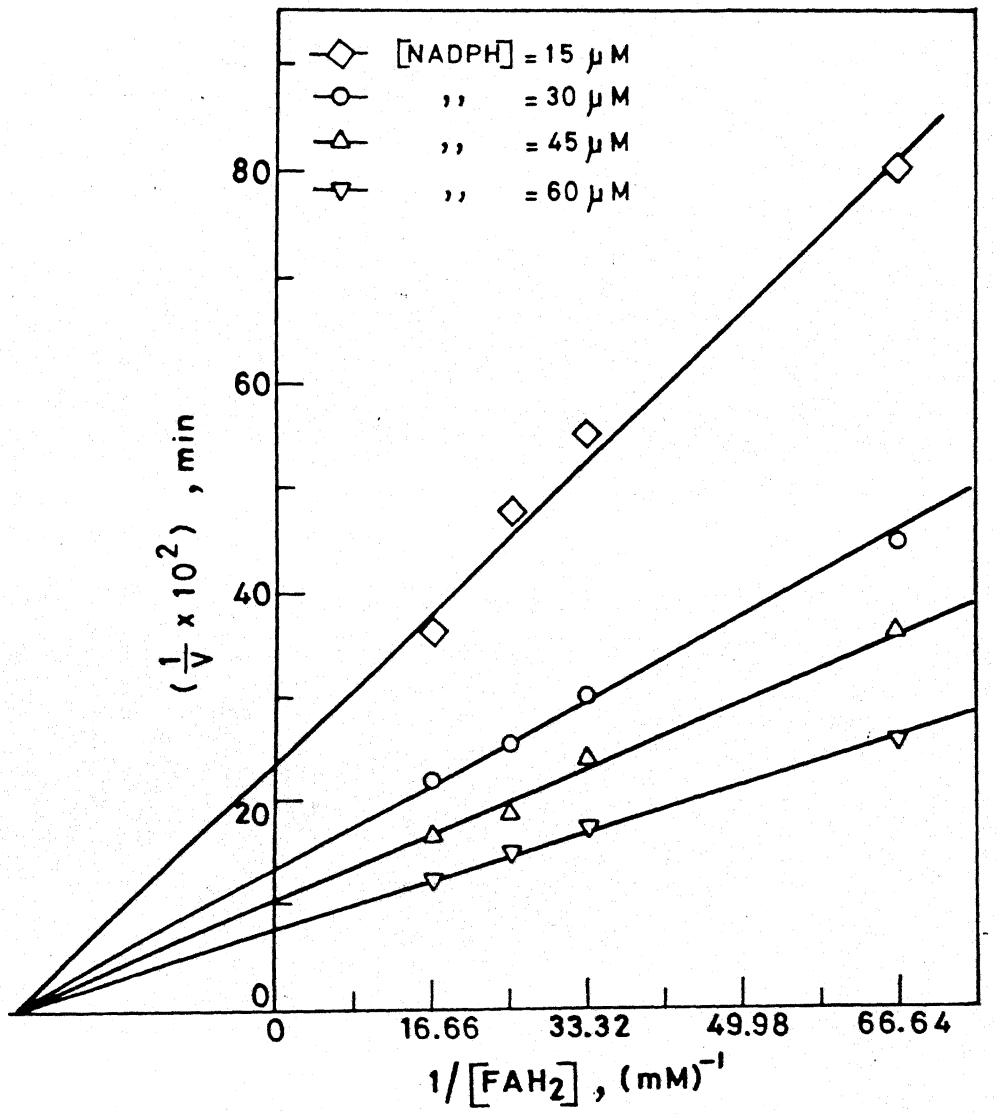


Fig.IV.8. Double reciprocal plots for initial DHFR rate with FAH_2 concentrations in CTAB/chloroform: isooctane (1:1, v/v) at different NADPH concentrations and w_o 14.44, pH 7.0, 100 mM CTAB.

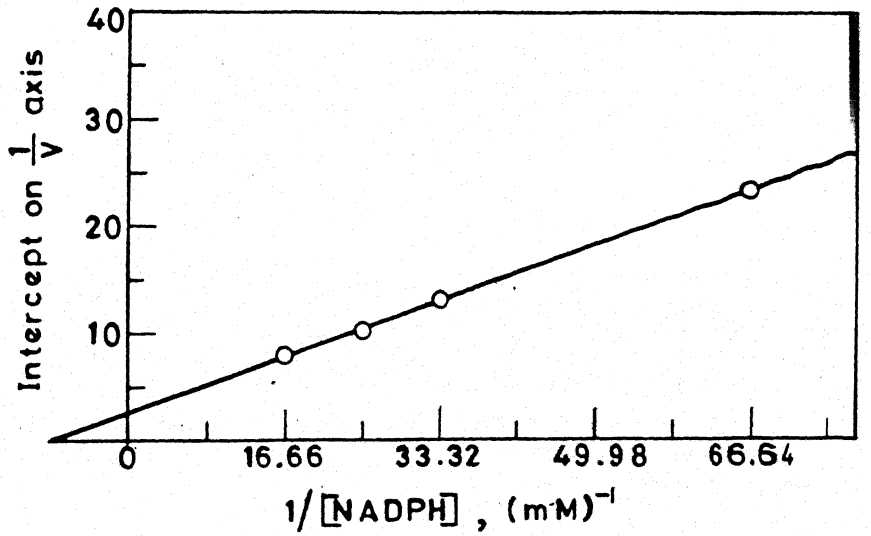


Fig. IV.9(b)

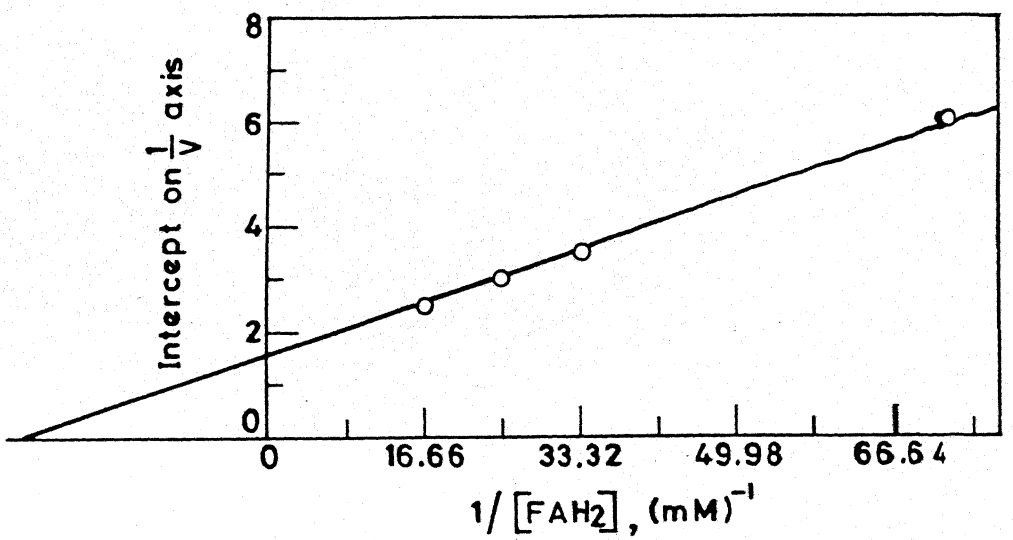


Fig.IV.9(a)

Fig.IV.9(a) Intercept on 1/v axis of Fig. IV.7 plotted as a function of inverse FAH_2 concentrations.

Fig.IV.9(b) Plot of intercept on1/v axis of Fig. IV.8 with reciprocal of NADPH concentrations.

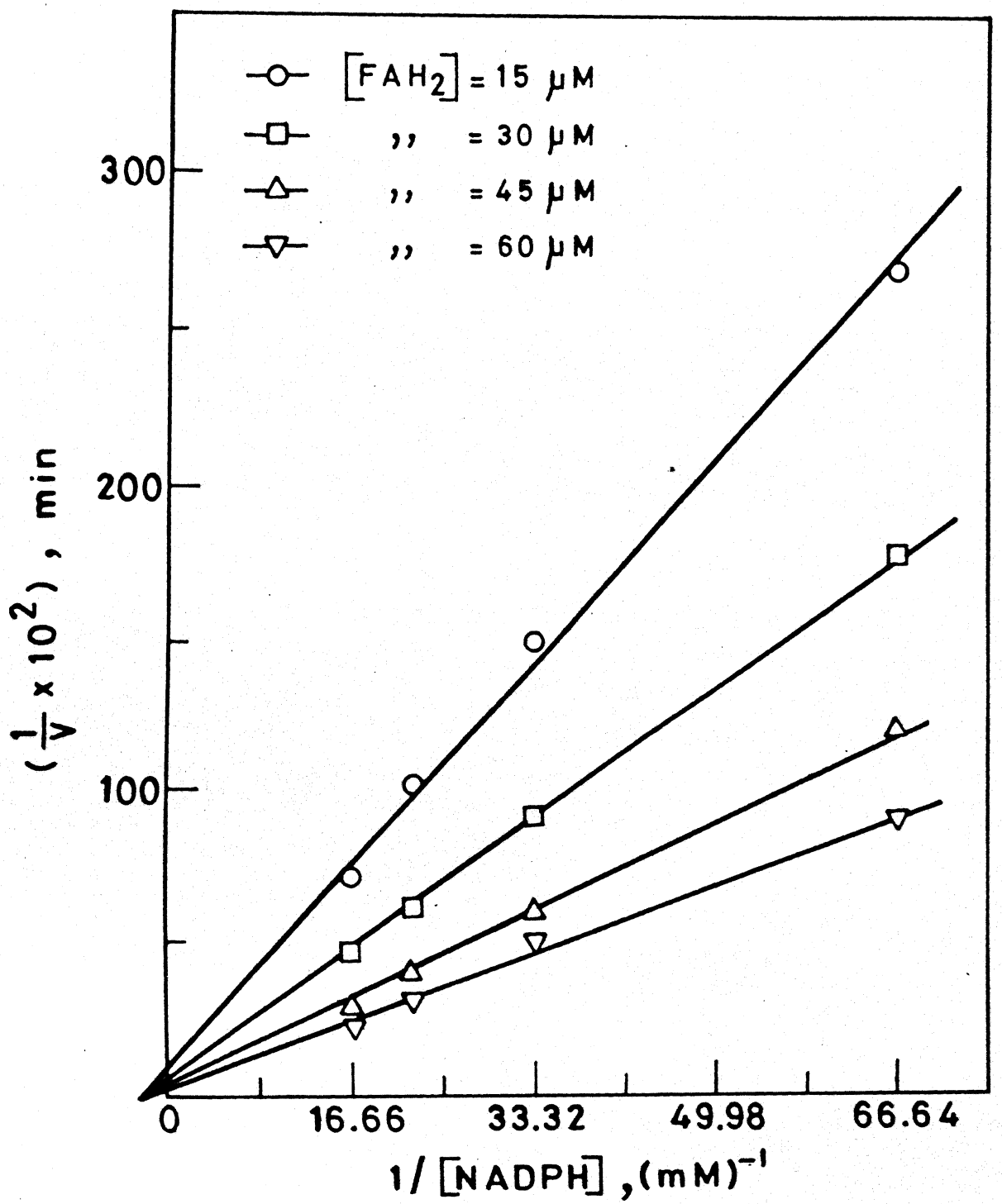


Fig.IV.10. Lineweaver-Burk plots for initial rate of DHFR with NADPH concentrations in 100 mM CTAB in chloroform:isooctane (1:1, v/v) at different concentrations of FAH_2 and w_o 7.22, pH 7.0.

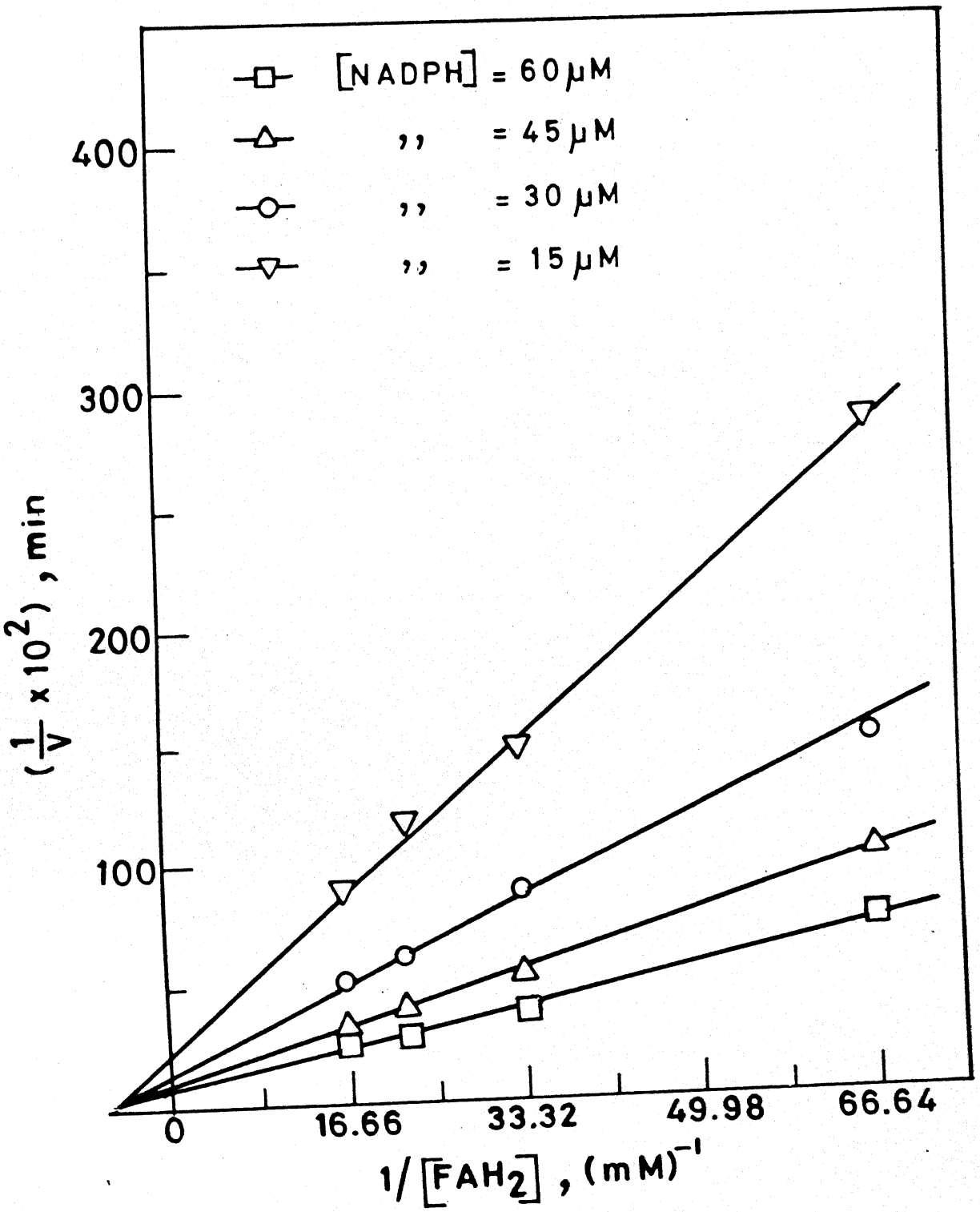


Fig.IV.11. Lineweaver-Burk plots for initial DHFR rate with FAH_2 concentrations in CTAB reverse micellar system at different NADPH concentrations and w_o 7.22, pH 7.0 and 100 mM CTAB.

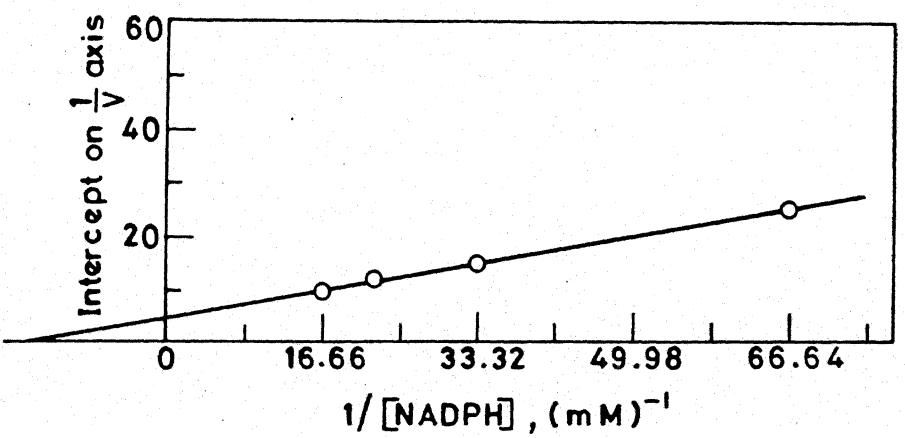


Fig.IV.12(b)

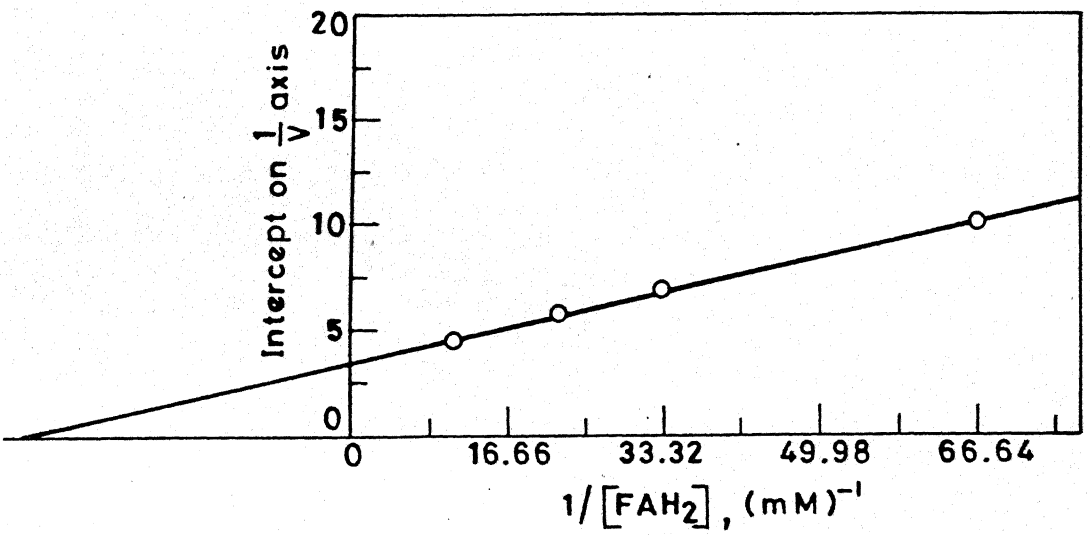


Fig.IV.12(a)

Fig.IV.12(a) Intercept on 1/v axis of Fig. IV.10 plotted as a function of inverse FAH_2 concentrations.

Fig.IV.12(b) Intercept on 1/v axis of Fig. IV.11 plotted as a function of reciprocal of NADPH concentrations.

at two w_o values viz. 14.44 and 7.22. The value of these kinetic parameters are reported in overall volume as well as in the water pool of reverse micelles.

Fig. IV.13 and IV.14 show the Lineweaver-Burk plots for LDH catalyzed reaction at w_o = 30.55, pH 7.0 and 100 mM CTAB concentration. K_m^{NADH} and $K_m^{Na\ Pyruvate}$ have been calculated from the secondary plots(IV.15). LDH catalyzed reaction was further studied at a lower water pool namely at w_o = 16.66, pH 7.0 and 100 mM CTAB concentration and the data in the form of double reciprocal plots is shown in Fig. IV.16 and IV.17. K_m values at this water pool were obtained from Fig. IV.18. The kinetic and binding properties of LDH obtained from the data of Figs. IV.13 to IV.18 are given in Table IV.2.

Effect of substrate concentrations on malate dehydrogenase activity shows that MDH also follows Lineweaver-Burk kinetics for both NADH and oxaloacetate (Fig. IV.19 and IV.20). Michaelis constant for the MDH have been calculated from Fig. IV.21. Kinetic and other parameters for MDH catalysed reaction are summarized in Table IV.3. These results on the dependence of substrate concentration on enzyme activity show that in general these enzymes (DHFR, LDH and MDH) follow initial velocity pattern in reverse micellar medium.

The values of $(K_m)_{ov}$ determined for these enzymes seem to be closer to the value obtained in water whereas $(K_m)_{wp}$ shows very high value compared to those in water. Since K_m is a good measure of the dissociation constant of the enzyme-substrate complex,[5,9] $(K_m)_{ov}$ indicates that the stability of the ES complex

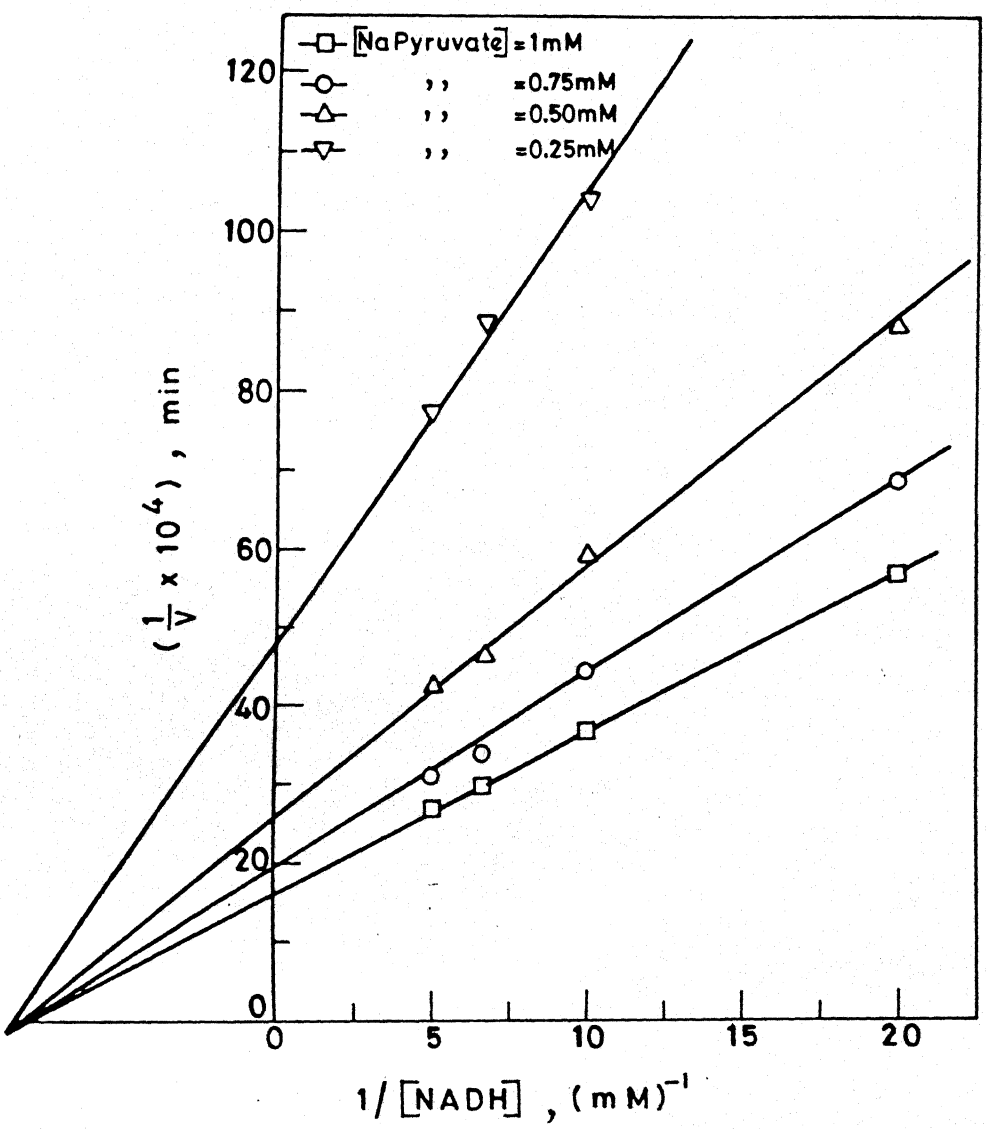


Fig.IV.13. Double reciprocal plots for initial rate of LDH with NADH concentrations in CTAB reverse micellar system at different Na pyruvate concentrations and w_o 30.55, pH 7.0 and 100 mM CTAB.

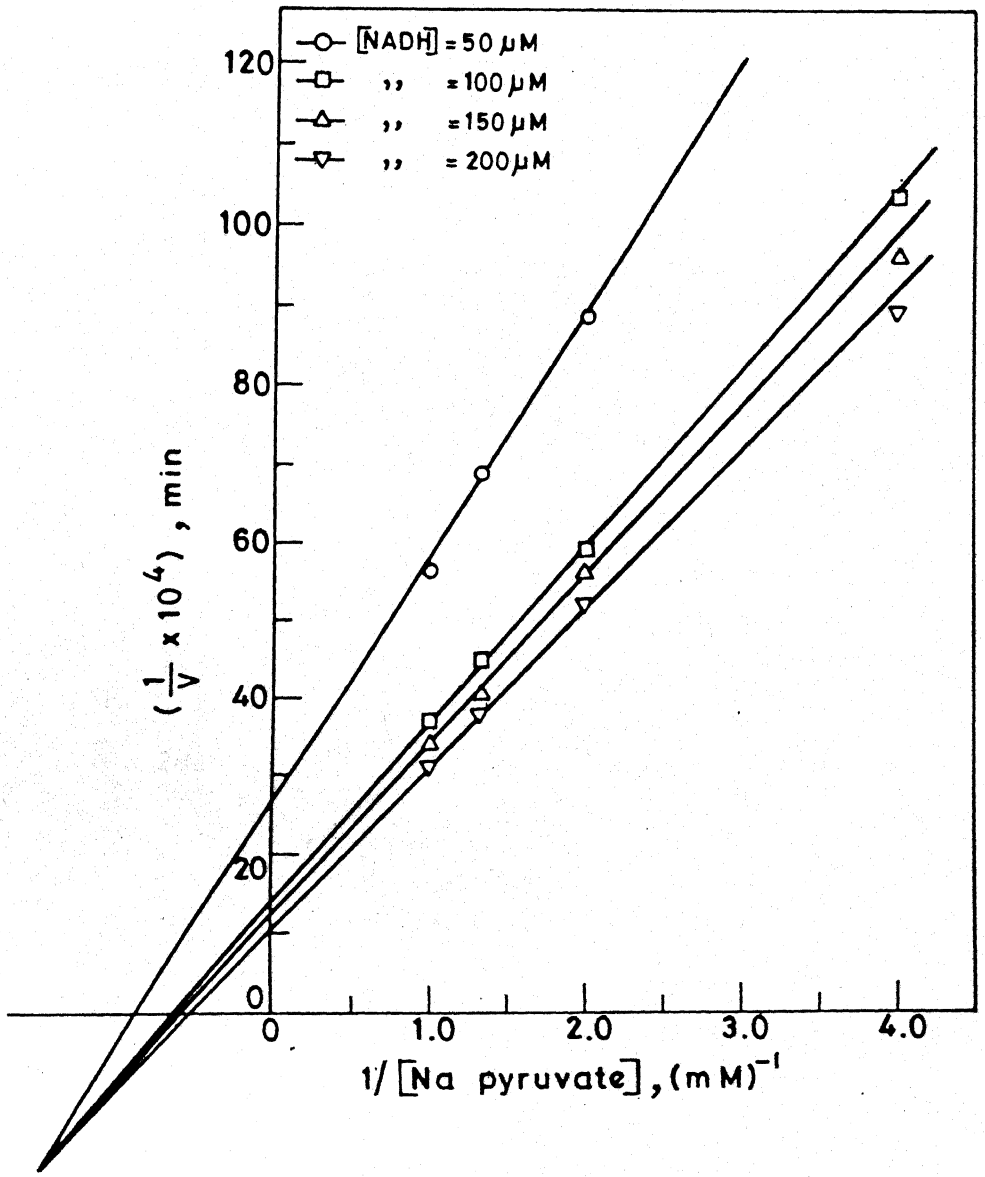


Fig.IV.14. Lineweaver-Burk plots for initial rate of LDH with Na pyruvate concentration in CTAB/chloroform:iso-octane (1:1, v/v) at different concentrations of NADH and w_o 30.55, pH 7.0 and 100 mM CTAB.

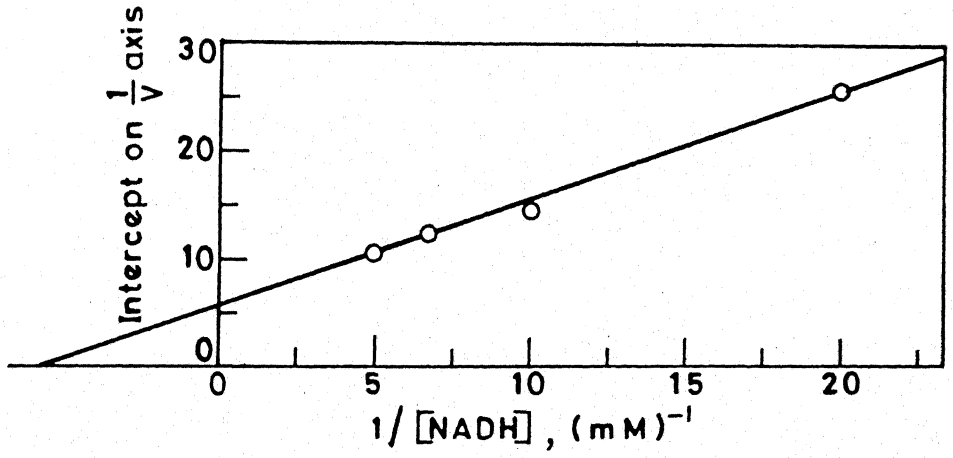


Fig.IV.15(b)

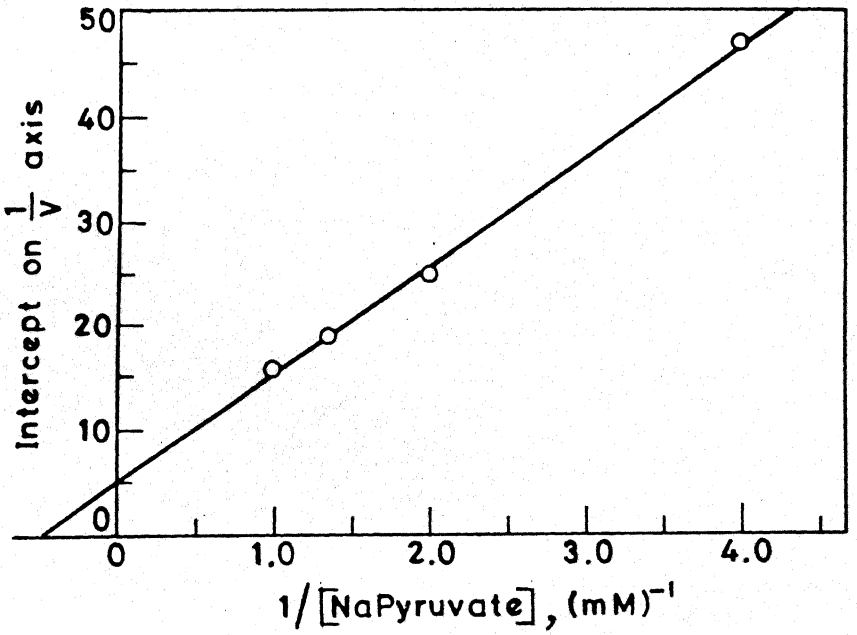


Fig.IV.15(a)

Fig.IV.15(a) Intercept on 1/v axis of Fig. IV.13 plotted as a function of reciprocal of Na pyruvate concentrations.

Fig.IV.15(b) Secondary plot of intercept on 1/v axis of Fig. IV.14 vs reciprocal of NADH concentrations.

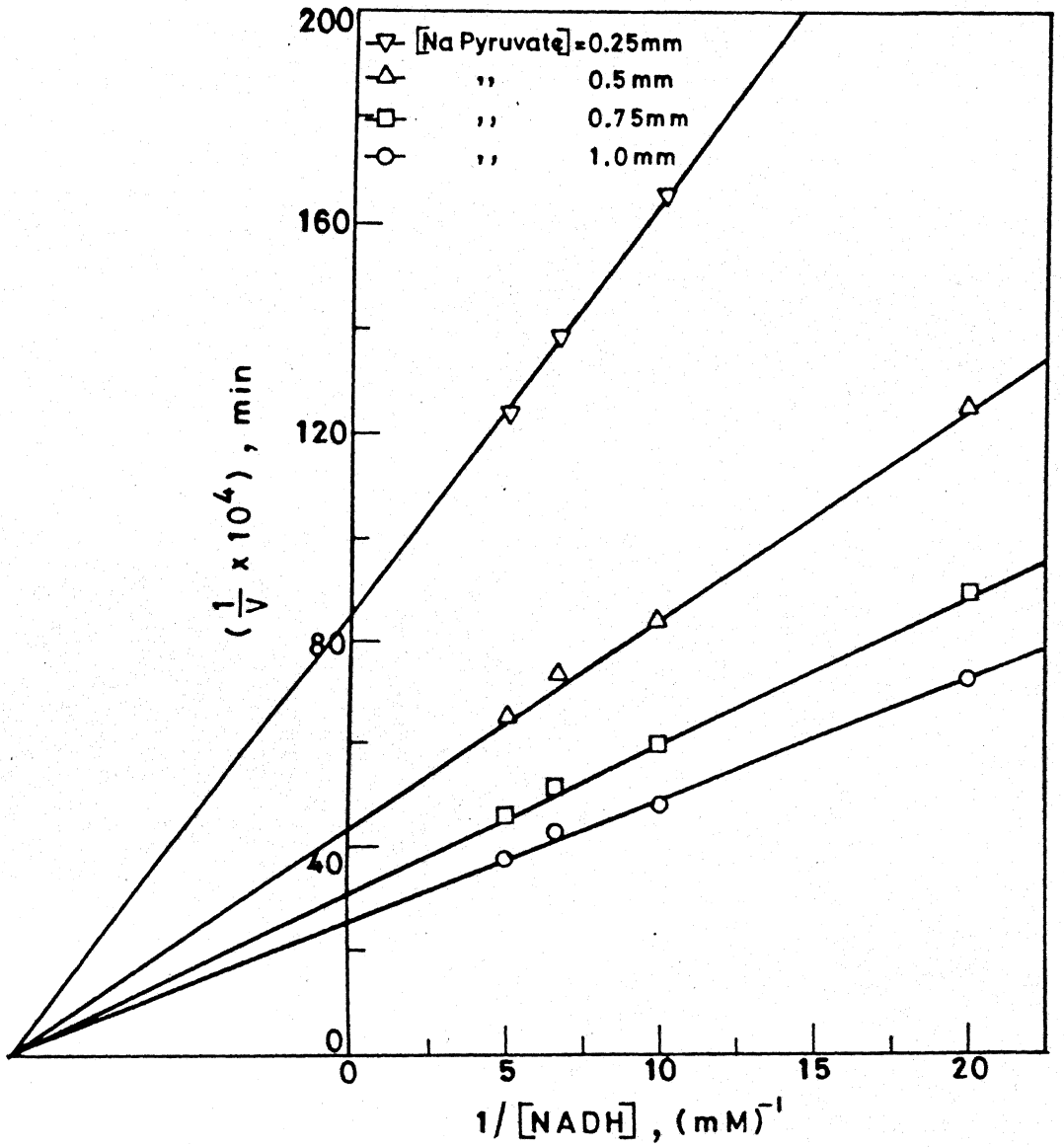


Fig.IV.16. Lineweaver-Burk plots for initial LDH rate with NADH concentrations in CTAB reverse micellar system at different Na pyruvate concentration and w_o 16.66, pH 7.0 and 100 mM CTAB.

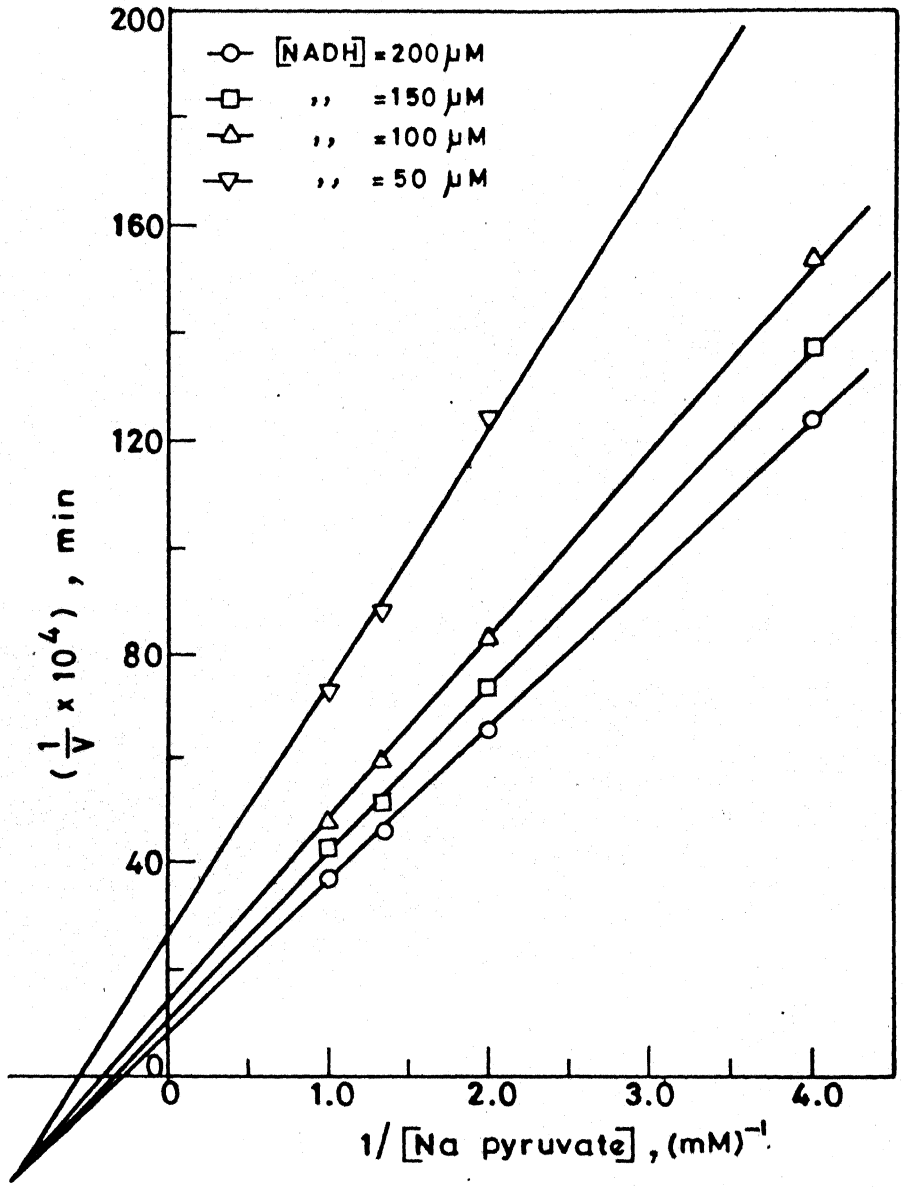


Fig.IV.17. Lineweaver-Burk plots for initial rate of LDH with Na pyruvate concentrations in CTAB/chloroform:isooctane (1:1, v/v) at different NADH concentration and w_o 16.66, pH 7.0 and 100 mM CTAB.

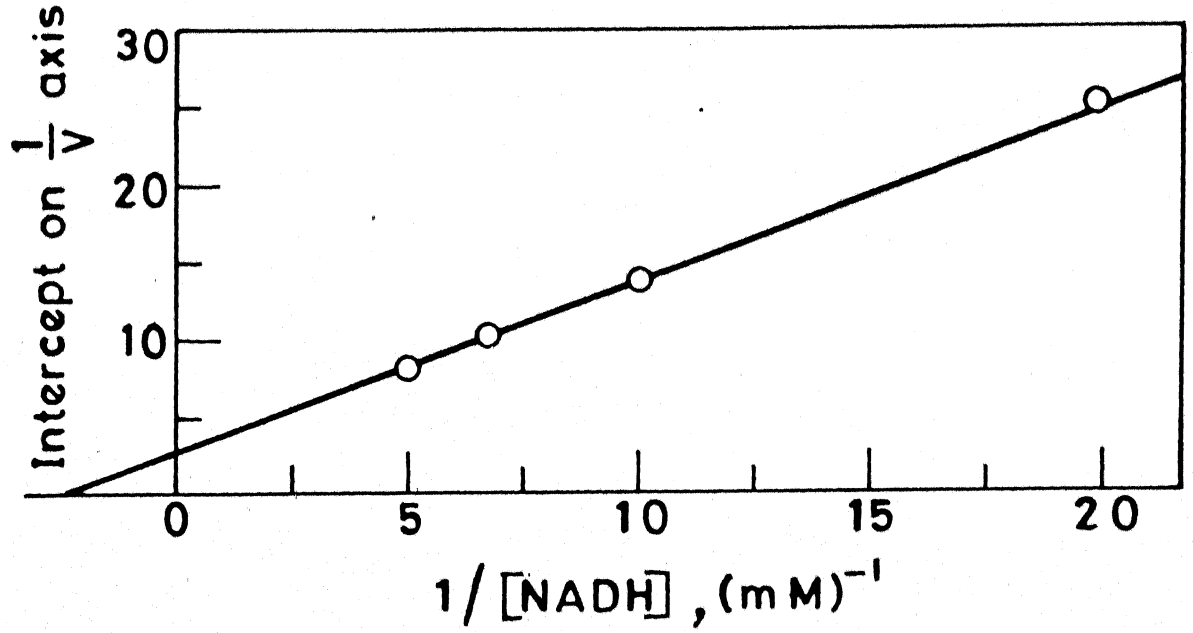


Fig.IV.18(b) Intercept on 1/v axis of Fig. IV.17 plotted as a function of NADH concentration.

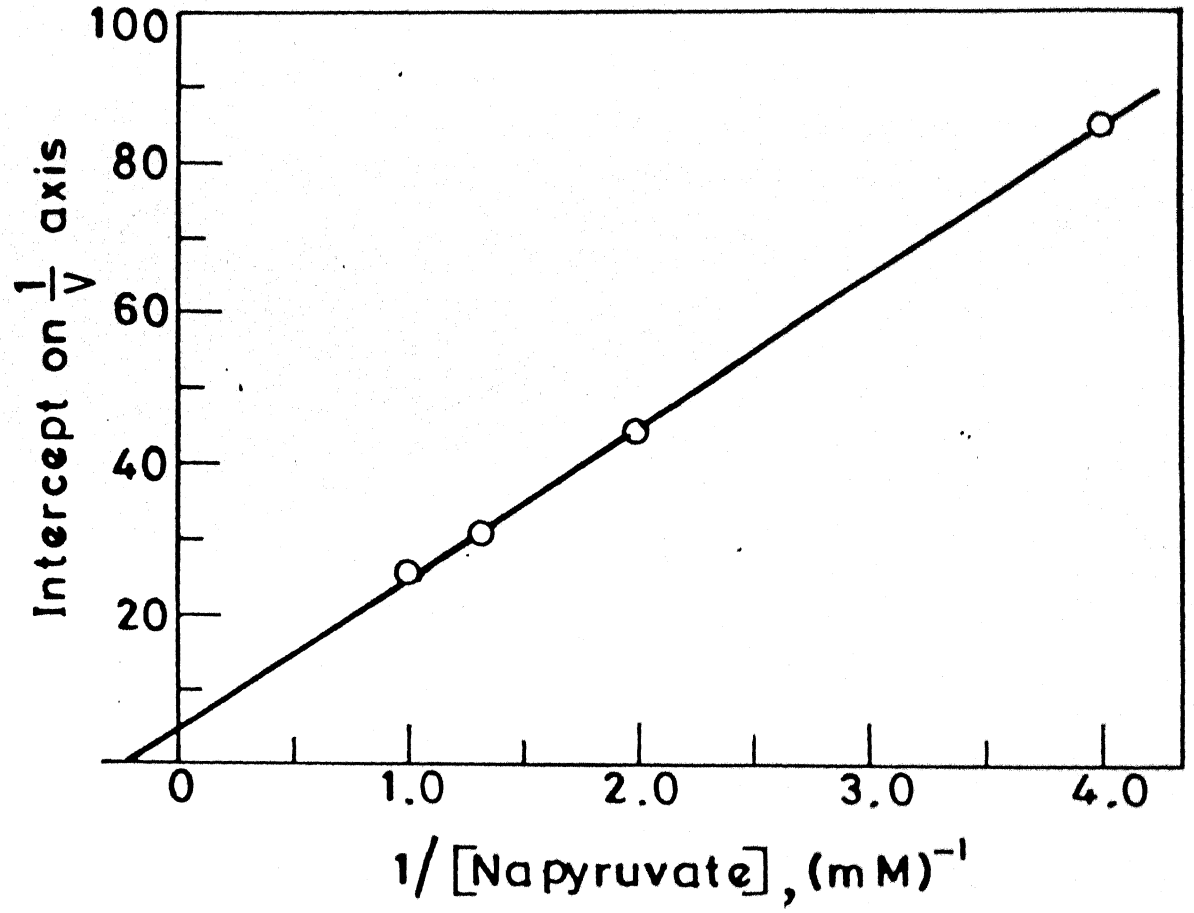


Fig.IV.18(a) Secondary plot of intercept on 1/v axis of Fig. IV.16 vs inverse of Na pyruvate concentrations.

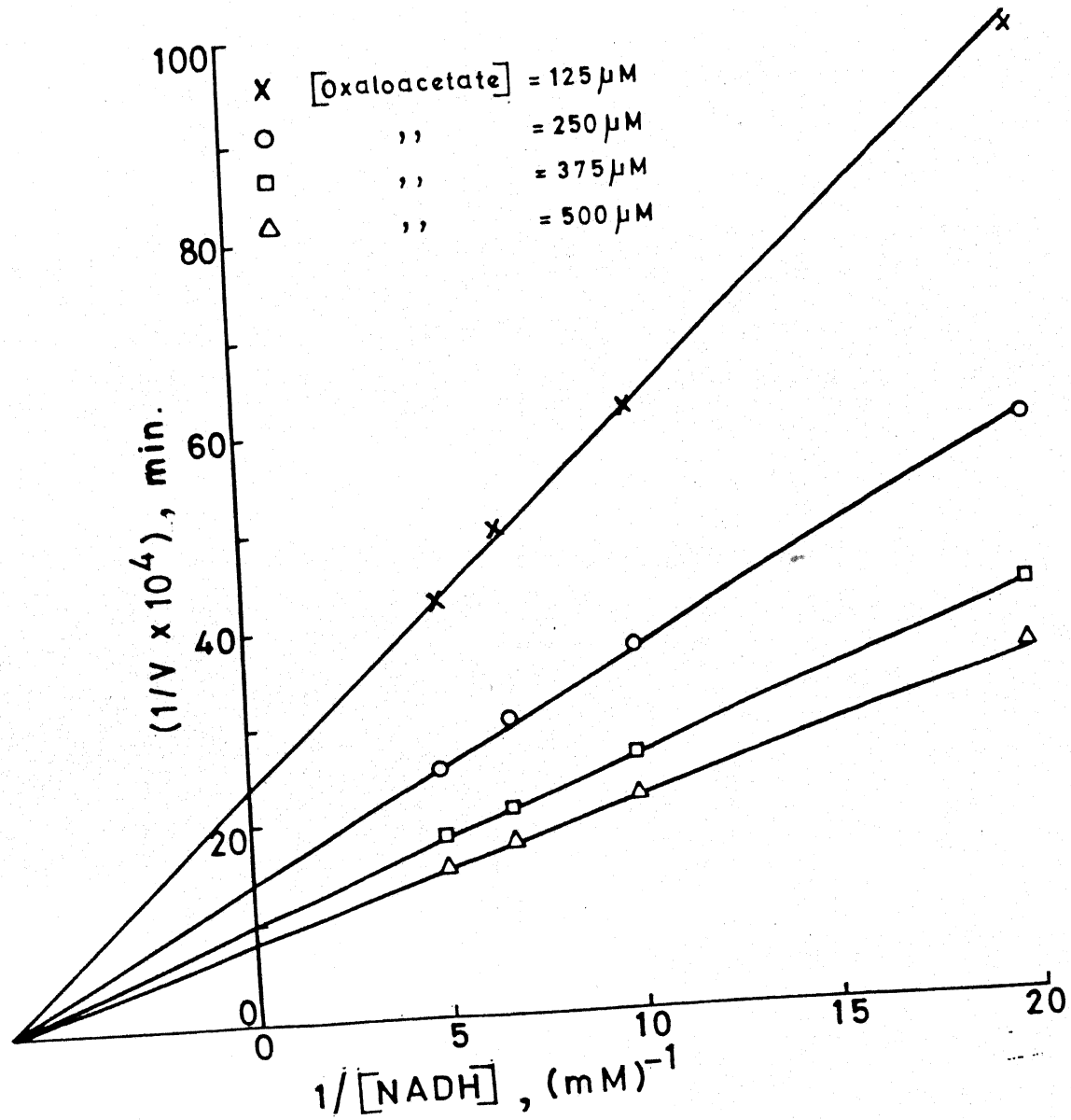


Fig.IV.19. Double reciprocal plots for initial rate of MDH with NADH concentration in 100 mM CTAB in chloroform-isooctane at different oxaloacetate concentration and w_o 25.55, pH 10.3.

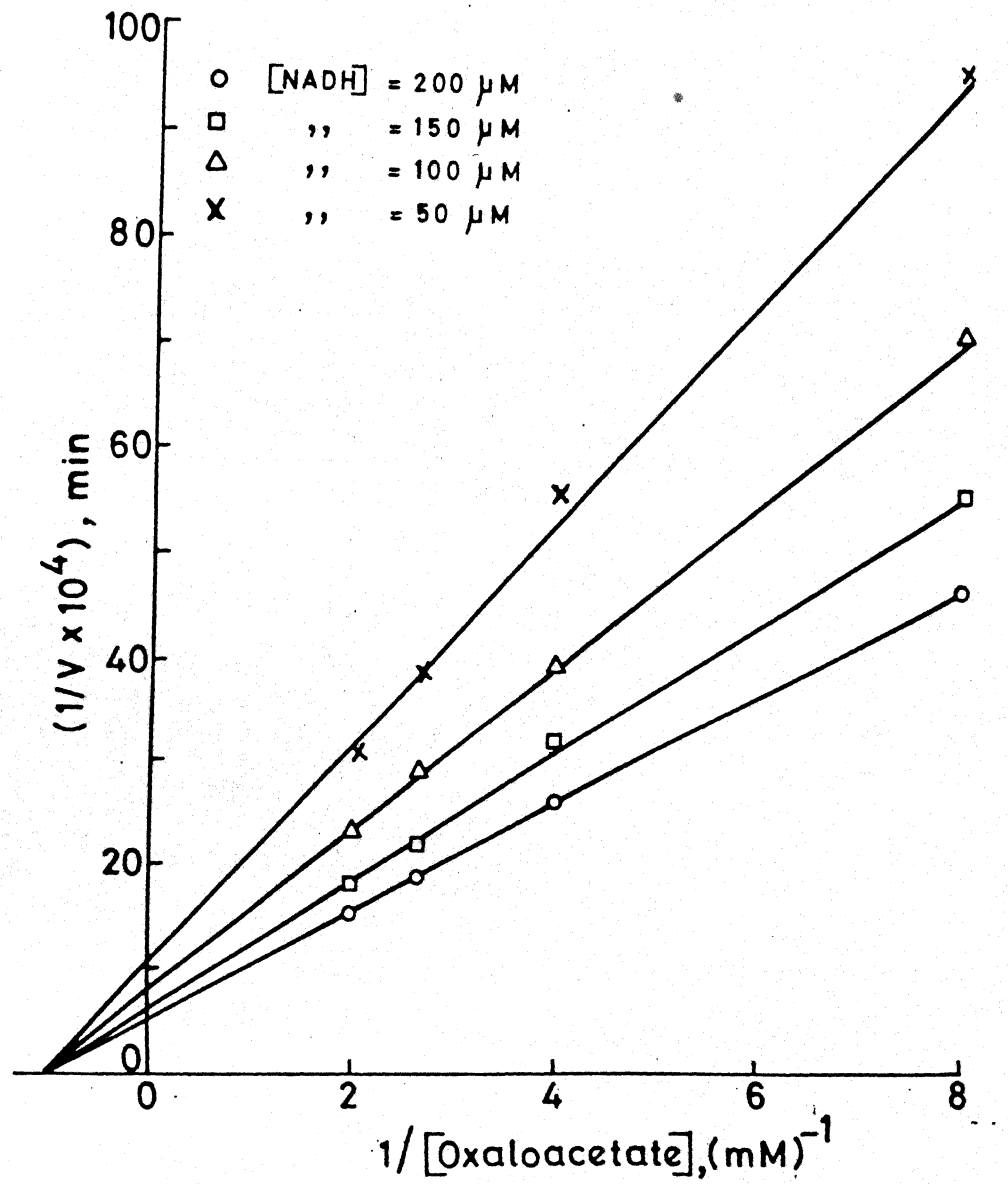


Fig.IV.20. Lineweaver-Burk plots for initial MDH rate with oxaloacetate concentration in CTAB micellar sys at different NADH concentrations and w_o 25.55, pH 10.3 and 100 mM CTAB.

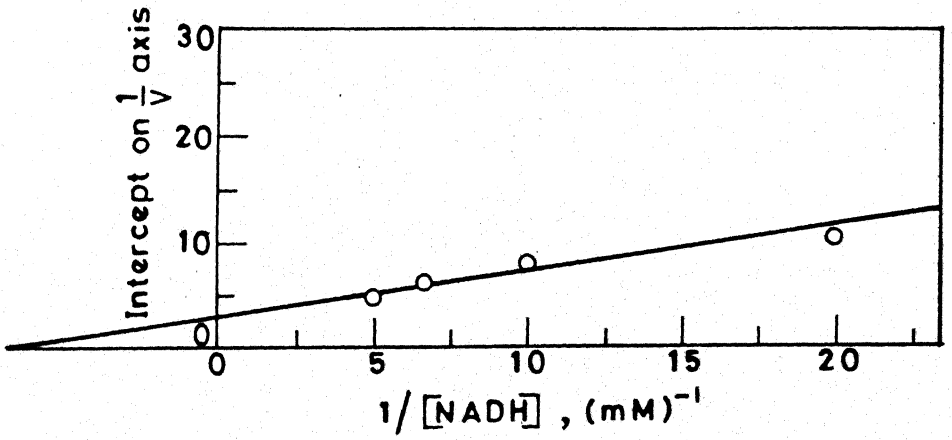


Fig.IV.21(b)

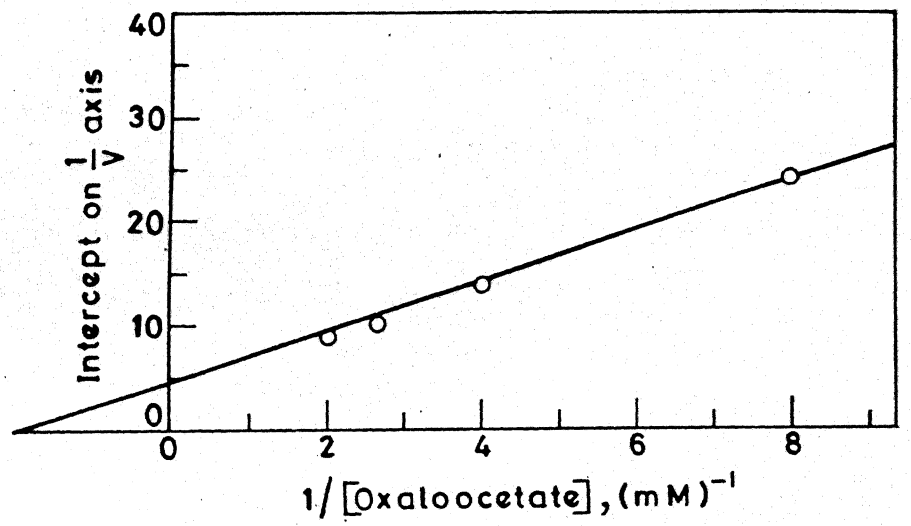


Fig.IV.21(a)

Fig.IV.21(a) Intercept on 1/v axis of Fig. IV.19 plotted as a function of oxaloacetate concentrations.

Fig.IV.21(b) Intercept on 1/v axis of Fig. IV.20 plotted as a function of NADH concentrations.

remains almost unaltered in reverse micelles while $(K_m)_{wp}$ shows that the ES complex is considerably destabilized as compared to that in aqueous solution.

In the case of DHFR, the comparison of data (Table IV.1) shows that on reducing the water content of the water pool, $(K_m)_{ov}$ and V_{max} also reduce while $(K_m)_{wp}$ and K_d (both overall and water pool) show increase in their values. Since $(K_m)_{ov}$ seems to be valid K_m in reverse micelles (reverse micellar solution behaves as a homogeneous solution, therefore substrate concentration is considered for overall volume) the decrease in $(k_m)_{ov}$ at low waterpool shows that ES complex is significantly stabilized as compared to that in higher water pools.

Michaelis constants determined for LDH show that $(K_m)_{ov}$ values are comparable to values of K_m obtained in aqueous solution whereas $(K_m)_{wp}$ values are an order of magnitude higher (Table IV.2). The K_m values obtained at two different w_o values viz w_o = 30.55 and 16.66, show that enzyme at higher water content (w_o = 30.55) displays low K_m and higher V_{max}. Low K_m indicates that ES complex is more stabilized at higher w_o value.

The K_m values obtained for MDH indicate that $(K_m)_{aqueous}$ is less than $(K_m)_{ov}$ and much less than the $(K_m)_{wp}$ (Table IV.3) the ES complex in micellar media is destabilized as compared to the aqueous medium.

Present study shows that in all the enzymes $(K_m)_{aqueous}$ is slightly smaller than the $(K_m)_{ov}$, where it is more than an order

TABLE IV.1. Kinetic and binding properties of dihydrofolate reductate (DHFR)

System	CTAB reverse micelle w_o = 14.44, pH≅7.0	CTAB reverse micelle w_o = 7.22, pH ≅ 7.0	Water pH=7.0
$K_m^{NADPH}(ov)^*$, mM	0.120	0.064	0.007
$K_m^{NADPH}(wp)^{\neq}$, mM	4.620	4.920	-
$K_m^{FAH_2}(ov)$, mM	0.038	0.028	0.010
$K_m^{FAH_2}(wp)$, mM	1.440	2.140	-
$K_d^{NADPH}(ov)$, mM	0.200	0.360	-
$K_d^{NADPH}(wp)$, mM	7.690	27.690	-
V_{max}, μM min^{-1} (mg enzyme)$^{-1}$	8.110	4.475	8.110

* ov = overall
≠ wp = waterpool

TABLE IV.2. Kinetic and binding properties of lactate dehydrogenase (LDH).

System	CTAB reverse micelle w_o = 30.55, pH^o = 7.0	CTAB reverse micelle w_o = 16.66, pH^o = 7.0	water pH=7.0
$K_m^{NADH}(ov)^*$, mM	0.166	0.400	0.065
$K_m^{NADH}(wp)^{\neq}$, mM	3.018	13.330	-
$K_m^{Na\ pyruvate}(ov)$, mM	2.000	5.000	0.800
$K_m^{Na\ pyruvate}(wp)$, mM	36.360	166.660	-
$K_d^{NADH}(ov)$, mM	0.115	0.090	-
$K_d^{NADH}(wp)$, mM	2.100	3.000	-
V_{max}, $\mu M\ min^{-1}$ (mg enzyme)$^{-1}$	362.900	266.100	362.900

* ov = overall
≠ wp = waterpool

TABLE IV.3. Kinetic and binding properties of malate dehydrogenase (MDH).

System	CTAB reverse micelle w_o = 25.55, pH^o = 10.3	water pH=7.5
$K_m^{NADH}(ov)^*$, mM	0.150	0.052
$K_m^{NADH}(wp)^{\neq}$, mM	3.260	-
$K_m^{Oxaloacetate}(ov)$, mM	0.500	0.034
$K_m^{Oxaloacetate}(wp)$, mM	10.870	-
$K_d^{NADH}(ov)$, mM	0.160	-
$K_d^{NADH}(wp)$, mM	3.480	-
V_{max}, $\mu M\ min^{-1}$ (mg enzyme)$^{-1}$	661.300	826.600

* ov = overall
≠ wp = waterpool

of magnitude smaller than $(K_m)_{wp}$. Similarly the value of $(K_m)_{ov}$ determined for other enzyme studied in reverse micelle namely α-chymotrypsin,[10], lysozyme,[11] horseliver alcohol dehydrogenase,[8] much more closer to $(K_m)_{aqueous}$. These results strongly support the concept that $(K_m)_{ov}$ is a valid K_m in reverse micellar media and it should be preferred in comparison to $(K_m)_{wp}$.

REFERENCES

1. Segel, I.H. (1975) Enzyme kinetics, John Wiley, New York.

2. Dixon, M., Webb, E.C. (1979) Enzyme IIIrd Ed., Longman, London

3. Scopes, R.K. (1982) Protein purification,Springer-Verlag, New York.

4. Ferst, A. (1977) Enzyme structure and mechanism, Freeman, San Francisco.

5. Stryer, L. (1975) Biochemistry, Freeman, San Francisco.

6. Levashov, A.V., Khmelnitski, Y.L., Klyachko, N.L. and Martinek, K. (1984) in surfactant in solution (Mittal, K.L. and Lindman, B., eds.) Vol. 2, pp. 1069-1091.

7. Martinek, K., Levashov, A.V., Klyachko, N.L., Pantin, V.I. and Berezin, I.V. (1981) Biochim. Biophys. Acta 657, 277-294.

8. Meier, P. and Luisi, P.L. (1980) J. Solid. Phase Biochem. 5, 269-282.

9. Luisi, P.L. Meier, P., Imre, V.E. and Pande, A. (1984) in Reverse Micelles (Luisi, P.L. and Straub B.E. Eds.) pp. 323-337, Plenum Press, New York.

10. Barbaric, S. and Luisi, P.L. (1981) J. Am. Chem. Soc. 103, 4239-4244.

11. Grandi, C., Smith, R.E. and Luisi, P.L. (1981) J. Biol. Chem. 256, 837-843.

was successfully solubilized in non-aqueous medium. The enzyme shows its full activity in the medium which was 100% of its value in aqueous buffer at its optimum conditions. These results with LDH indicate that not only simple and small enzymes but also large and complex enzymes can be solubilized in apolar solvents where they can retain their activity.

(6) For these enzymes in general it appears that enzyme activity is highly regulated by different parameters such as w_o, pH, surfactant concentration, substrate concentration etc.

(7) The studies show that these enzymes maintain their conformational integrity and subunit-subunit interaction in reverse micellar medium.

(8) The results of time dependent stability on these enzymes (DHFR, LDH and MDH) show that these enzymes are stable for certain period of time in reverse micelles and at some conditions a better stability in reverse micelles than in aqueous medium was achieved.

(9) The kinetic characteristics of these enzymes (DHFR, MDH and LDH) show that they obey Michaelis-Menten kinetics in this microheterogeneous medium upto specific concentration range of substrate or coenzyme.

(10) As in aqueous medium these enzymes (DHFR, MDH and LDH) follow same kind of initial velocity patterns in this non-aqueous medium.

(11) For these enzymes (DHFR, LDH and MDH) the Michaelis constants (K_m) in reverse micellar medium at different w_o were calculated from Lineweaver-Burk plots which are quite close to the value of K_m in aqueous medium. Since K_m is a good measurements of dissociation constant of enzyme-substrate (E-S) complex therefore K_m in micellar medium ($K_m^{micelle} \approx K_m^{aqueous}$) indicates that the stability of the E-S complex in reverse micellar media remains almost unaltered.

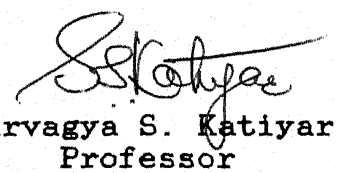
It seems that these enzyme (DHFR, LDH and MDH) retain their maximum activity, conformational integrity, stability and kinetic characteristics in the hostile medium of surfactant and organic solvent as both the surfactant and organic solvent, when they are used separately are poisons for enzymes. But the aggregation of surfactants in organic solvents protects enzymes from denaturation and provides a realistic picture of the enzyme behavior as this microheterogeneous environment is somewhat similar to cellular environment. Since reverse micelles have some features similar to those of biomembranes, therefore display of super activity by DHFR shows that enzyme <u>in vivo</u> may posses higher activity than actually found by <u>in vitro</u> studies in aqueous solutions.

Enzyme containing reverse micelles open new prospects for studies on membrane bound enzymes which are either unstable or loose some fraction of their activity in aqueous solution and on enzyme having water-insoluble substrates. Since enzyme containing reverse micelles have many biotechnological

application such as fine organic synthesis, synthesis of water insoluble compounds (steroids, lipids, fats etc.), peptide synthesis, separation and isolation of proteins, energy conversion processes, medical application etc. Hence presently investigated enzymes which are almost fully active or super active in microheterogeneous system, may find widespread use in enzyme mediated synthetic processes. It seems that this versatile medium for the study of enzyme reactions in vitro has tremendous potential and may help to extended the utility of enzymes in other area of science.

CERTIFICATE III

Certified that some of the research papers of Mr. Anil Kumar with me, have been published in the name Anil K. Awasthi. Both the names belong to the same person as Awasthi is the surname of Mr. Anil Kumar.



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LIST OF PUBLICATIONS

1. Behavior of enzymes in reverse micelles in non-aqueous solvents,
S.S. Katiyar, Anil K. Awasthi and Ajay Kumar,
Proc. Ind. Natl. Sci. Acad. (In press).

2. The phenomenon of super activity in dihydrofolate reductase entrapped inside reverse micelles in apolar solvents,
S.S. Katiyar, Anil K. Awasthi and Ajay Kumar,
FEBS Letters (Submitted).

3. Reverse micelles as a versatile medium for *in vitro* study of lactate dehydrogenase,
S.S. Katiyar, Anil K. Awasthi and Ajay Kumar,
Biochemistry International (Submitted).

4. Activity and stability of glutathione reductase in non-polar solvents in presence of surfactants,
Sarvagya S. Katiyar, Ajay Kumar and Anil K. Awasthi,
Biochem. Biophys. Res. Commun. (Submitted).

5. In vitro study of activity and kinetic characteristics of glutathione reductase in membrane like cellular environment,
Ajay Kumar, Anil Kumar and sarvagya S. Katiyar,
Biochim. Biophys. Acta (Submitted).

6. In vitro study of dehydrogenases in reverse micelles in apolar solvents: Activation of dihydrofolate reductase and lactate dehydrogenase,
Anil K. Awasthi and S.S. katiyar,
Proceedings Seventh International Symposium on Surfactants in Solution, Ottawa, Canada (Submitted).

7. Stabilization and super activity of yeast alcohol dehydrogenase in microheterogeneous medium of reverse micelles,
S.S. Katiyar, Tapas, K. De and Anil,K. Awasthi,
Proceedings Seventh International Symposium on Surfactants in Solution, Ottawa, Canada (Submitted).

8. Stabilization and kinetic behavior of dihydrofolate reductase in microheterogeneous medium comprising of surfactant in non-aqueous system,
Anil Kumar, Ajay Kumar and Sarvagya S. Katiyar,
(Manuscript under preparation).

9. Comprehensive study of lactate dehydrogenase in non-aqueous medium stabilized by surfactants,
Anil Kumar, Ajay Kumar and Sarvagya S. Katiyar,
(Manuscript under preparation).

10. Study of malate dehydrogenase in micro-captive environment generated by surfactants in apolar solvents,
Anil Kumar, Ajay Kumar and Sarvagya S. Katiyar,
(Manuscript under preparation).

11. Reverse micellar enzymology and its biotechnological applications,
Anil kumar, Ajay Kumar and Sarvagya S. Katiyar,
(Manuscript under preparation).

12. Study of reactions of carbocations with nucleophiles in microenvironment provided by reverse micelles in organic solvents,
Anil Kumar and Sarvagya S. Katiyar,
(Manuscript under preparation).

13. Catalytic and inhibitory effects on the reaction of carbonium ions with nucleophiles in micellar medium,
Anil Kumar, and Sarvagya S. Katiyar,
(Manuscript under preparation).

PRESENTATIONS

1. Catalytic activity of glutathione reductase and malate dehydrogenase in inverted micelles in non-aqueous solvents,
S.S. Katiyar, Anil K. Awasthi and Ajay Kumar,
IIIrd National Conference on Surfactants, Emulsions and Biocolloids, Aligarh, India, 1987.

2. Reactions of N-alkyl substituted triarylmethyl carbonium ions with nucleophiles in reverse micelles and normal micelles,
Sarvagya S. Katiyar and Anil K. Awasthi,
Eighth IUPAC Conference on Physical Organic Chemistry, Tokyo, Japan, 1986.

3. Effect of normal and reverse micelles on the reaction of triphenylmethane carbocations with phenoxide and hydroxide,
Anil K. Awasthi and Sarvagya S. Katiyar,
IIIrd National Conference on Surfactants, Emulsions and Biocolloids, Aligarh, India, 1987.

VITAE

The author was born on December 1, 1960 at Kanpur (U.P.), India. He passed the "High School Examination" conducted by U.P. Board, Allahabad in 1976 from Saraswati Vidya Mandir Inter college, Sikandra, Kanpur. After passing the "Intermediate Examination" conducted by the same board from Chacha Nehru Inter College, Kanpur, in 1978, he obtained his B.Sc. and M.Sc. degrees in 1980 and 1982 respectively from P.P.N College, Kanpur, affiliated to the Kanpur University .

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